SECTION I
REMARKS

Regarding the Amendments

No claims have been amended by the present amendment. As such, the claims are pending as submitted with the Response mailed October 11, 2007. Thus, upon entry of the Response, claims 1-21 will remain pending, of which claims 12-21 are withdrawn.

Claim Rejections Under 35 U.S.C. § 112, 1st paragraph, enablement

The examiner has maintained the rejection of claims 1-11 under 35 U.S.C. §112, first paragraph, as lacking enablement. Specifically, in the Final Office Action mailed January 9, 2008 the examiner alleges that the claims lack enablement because: 1) the art of producing a transgenic animal with a given phenotype is unpredictable (page 3), and 2) the art suggests that a double transgenic comprising CD4 and CCR5 or CXCR4 does not represent a model for human HIV-1 infection as taught by Sawada et al. and Browning et al. (page 4). Applicants respectfully disagree.

The examiner initially alleges that the claims are non-enabled because the art of producing a transgenic animal with a given phenotype is unpredictable. The examiner's argument is based on the allegation that "generating a transgenic rat is not the issue of enablement, the issue is generating a transgenic rat with a predictable phenotype, (e.i. [sic] – results in HIV infection)." (Office Action mailed January 9, 2008, p. 3.)

It is well established that "[t]he test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." (MPEP 2164.01) The present specification provides for transgenic rats, as set forth in the specification and the examples, and as acknowledged by the examiner. However, the examiner asserts that since example 12 is a prophetic example, "an artisan would not know how and if production of the transgenic rat taught in the specification would result in a model of human HIV infection." (Office Action mailed January 9, 2008, page 4.) It is respectfully submitted that the proper test for enablement is whether one so skilled in the art could reasonably make the transgenic rat that is taught in the specification as a model of human infection exhibiting HIV-1 binding without undue experimentation.

Clearly, the specification teaches one of skill in the art how to make a rat transgenic for HIV-1 (Examples 1-10), a rat transgenic for human CD4 (Example 11) and a double transgenic rat (Example 12). It is applicants' position that one of skill in the art could utilize such rats as models of human HIV infection without undue experimentation.

The examiner's attention is respectfully drawn to Exhibit A, an article titled "Progress Toward a Human CD4/CCR5 Transgenic Rat Model for De Novo Infection by Human Immunodeficiency Virus Type 1"[1] (hereinafter referred to as Keppler et al.) In the Keppler et al. article, the authors describe a CD4/CCR5 transgenic rat. The rat is generated by interbreeding hCD4 rats with hCCR5 rats to yield transgenic rats coexpressing hCD4 and hCCR5. Interbreeding of rats transgenic for a single gene, to generate a double transgenic rat is disclosed in Example 12 of the present application. Infection studies utilizing the rats generated in Keppler et al. showed that CD4 positive T lymphocytes, macrophages, and microglia from the hCD4/hCCR5 transgenic rats were highly susceptible to infection by HIV-1 R5 viruses. Keppler et al. also showed that primary rat macrophages and microglia from double-transgenic rats could be productively infected by various recombinant and primary R5 strains of HIV-1. Additionally, after the transgenic rats were systemically challenged with HIV-1, it was found that the lymphatic organs contained episomal 2-long terminal repeat (LTR) circles, integrated provirus, and early viral gene products, demonstrating susceptibility to HIV-1 *in vivo*. Low-level plasma viremia early in infection was also seen in the transgenic rats. All of these findings indicate that a double transgenic hCD4/hCCR5 rat is susceptible to HIV-1 *in vivo* and thus is useful as a model of HIV-1 infection in humans.

Additionally, at least a portion of the hypothesis tested by Keppler et al. was supported by and developed from a 2001 article, titled "An HIV-1 transgenic rat that develops HIV-related pathology and immunologic dysfunction"[2] (hereinafter referred to as Reid et al.), on which the present inventors are included as co-authors. (See Keppler et al., p. 732.) A copy of this paper is included herewith as Exhibit B. The Reid et al. paper reports Examples 1-9 of the present application and reports the resulting clinical manifestations similar to humans infected with HIV-1. Such findings were utilized by the authors of Keppler et al. in the development of the double transgenic hCD4/hCCR5 rat reported therein.

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[1] Keppler, O.T., et al., *J. Exp. Med.*, (March 18, 2002), vol. 195, no. 6, 719-736.
[2] Reid, W., et al., *PNAS*, (2001), vol. 98, no. 16, 9271-9276.

A more recent reference[3], including Keppler as an author and provided herewith as Exhibit C, demonstrates use of the double transgenic rats of Keppler et al. as models for HIV infection and utilizes these rats in evaluation of possible antiviral compounds. As such, transgenic rats are shown as useful models of human HIV-1 infection.

It is submitted that the above-cited references are not provided as "later dated publication[s]... [to] supplement an insufficient disclosure in a prior dated application to make it enabling," (MPEP 2164.05(a)), but are provided as evidence of the state of the art at the time of filing of the present application. References published subsequent to the present application show that those of skill in the art, relying on the results published by the present inventors regarding the HIV-1 transgenic rat, have generated a rat doubly transgenic for human CD4 and CCR5, utilizing a interbreeding method as specifically described in Example 12 of the present application. Additionally, those of skill in the art have tested such doubly transgenic rat for susceptibility to HIV-1 and have evaluated use of such rat as a model for evaluation of anti-HIV compounds. As such, the specification, as filed, is enabling for claims reciting generating a transgenic rat with a predictable phenotype, that of resulting in HIV infection and useful as a model of human HIV-1 infection.

The examiner also alleges that the claims are non-enabled because the art suggests that a double transgenic comprising CD4 and CCR5 or CXCR4 does not represent a model for human HIV-1 infection as taught by Sawada et al. and Browning et al. Again the examiner's attention is respectfully drawn to Exhibit C by Goffinet et al. In that reference hCD4/hCCR5 transgenic rats were used as "a model for rapid quantitative, and predictive evaluation of anti-HIV compounds." *In vivo* and *in vitro* tests "provide[s] proof of principle that rats...recapitulate the antiviral potency and basic pharmacological properties of two different anti-HIV drugs that are widely used in HIV-infected patients." (Goffinet, et al., p. 1018.) As is shown in the Goffinet et al. reference, a double transgenic is a model for human HIV-1 infection, as described in the present application.

As set forth in detail above, all aspects of pending claims 1-11 rejected under 35 U.S.C. §112, first paragraph, as lacking enablement are enabled. Accordingly, withdrawal of the rejection of these claims is respectfully requested.

[3] Goffinet, C., et al., *PNAS*, (2007), vol. 104, no. 3, 1015-1020.

Claim Rejections Under 35 U.S.C. § 112, 1st paragraph, new matter

In the Office Action mailed January 9, 2008, the examiner has rejected claims 1-11 under 35 U.S.C. §112, first paragraph, as containing subject matter that would be considered new matter with regard to the recitation "wherein the transgenic rat is a model for human HIV-1 binding" in claim 1 of the application. Applicants respectfully disagree.

Claim 1, as pending, recites "A transgenic rat, whose genome comprises at least one copy of a transgene encoding at least a portion of a CD4 protein sufficient for binding to gp120...and wherein the transgenic rat is a model of human HIV-1 binding." Accordingly, the transgenic rat, as claimed, is a model of human binding by virtue of the ability of the transgenic expressed CD4 to bind gp120. Such binding is sufficiently described in the specification so as to satisfy the written description requirement of 35 U.S.C. §112, first paragraph.

In the "Background of the Invention" section of the application, the binding action present in HIV infection in humans is described. In particular,

> "[t]he gp120 molecule binds to the CD4 antigens on the surface of cells, in particular helper T cells. Once HIV is bound to CD4 via gp120, another env gene product, gp41, mediates fusion between the membranes of the cell and the virus allowing the core of the virus to enter the cell. Gp120, which is expressed on the plasma membrane of infected cells before virus is released, can bind to CD4 on another cell, initiating a membrane fusion event resulting in syncytia formation, and HIV genomes can be passed between the fused cells directly." (Specification, p. 2, l. 32-p. 3, l. 4.)

In the specification at page 21, the transgenic rat of the invention is described as comprising

> "a nucleic acid encoding a human CD4 protein, or a portion thereof sufficient for binding to gp120 and allow an HIV to infect a cell expressing such a protein, under the control of a CD4 promoter, enhancer, and optionally, silencer, e.g., from human, mouse or rat species." (Specification, page 21, ll. 13-17.)

Clearly, such binding abilities are the same. Therefore, the specification in its written description provides for a transgenic rat with binding abilities that are the same as the binding abilities of humans. As such, the rat may serve as a model of human binding.

Recitation of the language "wherein the transgenic rat is a model for human HIV-1 binding" in claim 1 of the application is supported by the specification and does not constitute new matter, as

defined by 35 U.S.C. §112, first paragraph. Withdrawal of the rejection is therefore respectfully requested.

Claim Rejections Under 35 U.S.C. § 112, 2nd paragraph, definiteness

In the Office Action mailed July 11, 2007, the examiner has rejected claims 2-10 under 35 U.S.C. §112, second paragraph as indefinite for recitation of "a model of HIV infection" in claim 2 and "a model of HIV binding" through the dependency of claim 2 on independent claim 1. Applicants respectfully submit that such recitation is fully clear and no way indefinite.

The transgenic rat of the invention is a model of human HIV-1 binding, as recited in claim 1. The Background of the Invention section provides that "[a]lthough human CD4 is essential for HIV infection, it is not sufficient." (Specification, p. 4.) As such, a rat transgenic for solely CD4 is not being claimed as a model of HIV infection. Claim 2, however, recites a transgenic rat of claim 1 with a genome that further comprises a transgene encoding for CCR5. It is clearly described in the Background of the Invention section that CD4 and CCR5 are co-receptors for HIV entry into cells, a step in human HIV infection (Specification, p. 4.) As such, the CD4/CCR5 double transgenic rat is a model of human HIV-1 infection.

Since the claimed transgenic is a model of both HIV-1 infection and binding, it is respectfully submitted that claim 2, and claims 3-10 dependent therefrom, are sufficiently definite to meet the requirements of 35 U.S.C. §112, second paragraph. As such, withdrawal of the indefiniteness rejection of claims 1 and 11 under 35 U.S.C. §112, second paragraph is respectfully requested.

CONCLUSION

Based on the foregoing, all of Applicants' pending claims 1-11 are patentably distinguished over the art, and are in form and condition for allowance. The Examiner is requested to favorably consider the foregoing and to responsively issue a Notice of Allowance.

The time for responding to the January 9, 2008 Office Action without extension was set at three months, or April 9, 2008. This response is therefore timely and no fees are believed to be due for the filing of this paper. However, should any fees be required or an overpayment of fees made, please debit or credit our Deposit Account No. 08-3284, as necessary.

If any issues require further resolution, the Examiner is requested to contact the undersigned attorney at (919) 419-9350 to discuss same.

Respectfully submitted,

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Enclosures:
Exhibit A [19 pgs.]
Exhibit B [7 pgs.]
Exhibit C [7 pgs.]

The USPTO is hereby authorized to charge any deficiency or credit any overpayment of fees properly payable for this document to Deposit Account No. 08-3284

EXHIBIT A

Progress Toward a Human CD4/CCR5 Transgenic Rat Model for De Novo Infection by Human Immunodeficiency Virus Type 1

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Abstract

The development of a permissive small animal model for the study of human immunodeficiency virus type (HIV)-1 pathogenesis and the testing of antiviral strategies has been hampered by the inability of HIV-1 to infect primary rodent cells productively. In this study, we explored transgenic rats expressing the HIV-1 receptor complex as a susceptible host. Rats transgenic for human CD4 (hCD4) and the human chemokine receptor CCR5 (hCCR5) were generated that express the transgenes in $CD4^+$ T lymphocytes, macrophages, and microglia. In ex vivo cultures, $CD4^+$ T lymphocytes, macrophages, and microglia from hCD4/hCCR5 transgenic rats were highly susceptible to infection by HIV-1 R5 viruses leading to expression of abundant levels of early HIV-1 gene products comparable to those found in human reference cultures. Primary rat macrophages and microglia, but not lymphocytes, from double-transgenic rats could be productively infected by various recombinant and primary R5 strains of HIV-1. Moreover, after systemic challenge with HIV-1, lymphatic organs from hCD4/hCCR5 transgenic rats contained episomal 2–long terminal repeat (LTR) circles, integrated provirus, and early viral gene products, demonstrating susceptibility to HIV-1 in vivo. Transgenic rats also displayed a low-level plasma viremia early in infection. Thus, transgenic rats expressing the appropriate human receptor complex are promising candidates for a small animal model of HIV-1 infection.

Key words: HIV-1 • transgenic rats • CD4 • CCR5 • macrophages

Introduction

New small animal models of HIV-1 disease are needed to complement present models in the study of viral pathogenesis, the screening of new drugs, and the testing of vaccine strategies. Limitations of existing animal models include the availability and high cost of nonhuman primates, the ab-

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sence or delayed progression to an acquired immunodeficiency syndrome in some of these models, or permissivity only for related retroviruses (1–3). Current xenotransplant models are informative about select aspects of HIV-1 pathogenesis (4, 5), but present neither a complete range of infected tissues nor the context of an intact immune response.

HIV-1 replication is subject to a variety of potent species-specific restrictions in cells from many nonprimate species (6–10). Over the past years, a number of advances have been made elucidating the molecular bases of such blocks to HIV-1 replication, and these discoveries have recharged efforts to develop transgenic small animal models permissive for HIV-1 infection in the context of an intact immune system. Regarding cellular entry, coexpression of hCD4 and a human chemokine receptor were shown to overcome the entry block in primary T lymphocytes from mice transgenic for either hCD4 and hCCR5 (11) or hCD4 and human CXCR4 (hCXCR4) (12), but these mouse cells exhibited very little or no productive infection. Another restriction to HIV-1 replication in mouse cells is the limited efficiency of the viral regulator, Tat, in activating transcription and transcript elongation from the long terminal repeat (LTR)* of HIV-1, which are normally crucial steps for vigorous viral replication. Recently, a novel Tat-interacting protein, human cyclin T1 (hCycT1) was identified (13). hCycT1 was shown to be crucial to the transcriptional block in mouse cells since expression of hCycT1 drastically enhanced transcriptional activity in mouse NIH 3T3 fibroblasts (13–19) and in primary lymphocytes from transgenic mice (unpublished data). However, 3T3 cells expressing hCD4, hCCR5, and hCycT1 were still unable to support the full HIV-1 replication cycle (16, 17). In this context, a viral assembly block in 3T3 cells was reported (16) that could pose a species-specific, post-transcriptional barrier to HIV-1 replication. Interestingly, this HIV-1 assembly block could be partially complemented by mouse–human heterokaryon fusions (17, 18) suggesting that these mouse fibroblasts lack a specific positive factor required for efficient virus assembly and release.

An alternate approach to small animal model development is the identification of other species that are less restricted for the HIV-1 replication cycle but nonetheless susceptible to transgenic manipulation. In certain rat cell lines, cellular entry constitutes the only absolute block to HIV-1 replication and this restriction can be overcome by coexpression of hCD4 and hCCR5 (19). We and others have identified quantitative and qualitative limitations in various aspects of the HIV-1 replication cycle in rat cell lines (17–19) that appear to be largely cell type–specific, rather than species specific. Importantly, in the context of infections with HIV-1 pseudotypes containing the vesicular stomatitis virus G protein (VSV-G), primary nontransgenic rat cells from the monocyte/macrophage lineage supported all postentry steps in the viral life cycle and secreted substantial levels of infectious virions (19). Furthermore, unlike native mouse and hamster cells, most rat-derived cells supported a robust HIV-1 LTR activity (17–19). This conclusion is also confirmed by a recent report showing that HIV-1 transgenic rats carrying an HIV-1 provirus with functional deletions in *gag* and *pol*, expressed gp120, Tat, and Nef proteins in spleen tissue (20). Although overexpression of hCycT1 thus appears to be nonessential for efficient LTR transactivation in rat cells, our data indicated that the activity of CycT1 may still be a quantitatively limiting factor in this species context (19).

In addition, rats offer several practical advantages for their use as an animal model for HIV-1 infection, including their short gestation time, size, and well-characterized immune system and central nervous system (CNS). In particular, rats have long been considered a valuable model for studying the development and function of the CNS and they are used extensively to study the molecular mechanisms underlying HIV-related CNS pathology (21–23). Rats have also been used frequently as a model organism for basic pharmacological studies, including pharmacokinetic and pharmacodynamic studies on current anti-HIV drugs (24–26). Furthermore, rat transgenesis can be performed with relative ease, thereby enabling the selective expression of human genes that may be essential for full realization of the HIV-1 replication cycle in this species.

In humans, T lymphocytes as well as cells from the monocyte/macrophage lineage, including microglia, are considered to be the most important sites of HIV-1 replication in vivo (27, 28). To mimic the pattern of susceptible tissues and viral pathogenesis in a transgenic rat model, these cell types must express the human transgenes essential for complementation of the HIV-1 replication cycle. Although HIV-1 entry can be mediated by hCD4 acting with one of several members of the human chemokine receptor superfamily or related coreceptors (29), hCCR5 appears to be the critical coreceptor required for transmission and establishment of an infection in humans. Key pieces of evidence in support of this are the almost exclusive presence of CCR5-dependent (R5) viruses in early HIV-1 disease (30) and the high degree of resistance to HIV-1 infection observed in exposed individuals homozygous for a 32-basepair deletion in their CCR5 gene (Δ32 *CCR5*), which prevents the presentation of hCCR5 at the cell surface (31).

Here we report the generation of transgenic rats that coexpress hCD4 and hCCR5 on $CD4^+$ T lymphocytes, macrophages, and microglia. Coexpression of these human transgenes rendered primary rat cells permissive for infection by R5 viruses. In ex vivo cultures, cells of the monocyte/macrophage lineage from these animals could be productively infected by various R5 viruses at levels one to two orders of magnitude higher than those described for comparable transgenic mouse models (11, 12). In vivo, hCD4/hCCR5 transgenic rats challenged systemically

Abbreviations used in this paper: AZT, zidovudine; CNS, central nervous system; CycT1, cyclin T1; Env, envelope; ID, rodent identifier; LTR, long terminal repeat; MIP, macrophage inflammatory protein; MOI, multiplicity of infection; RANTES, regulated upon activation, normal T cell expressed and secreted; RTV, Ritonavir; SIV, simian immunodeficiency virus; VSV-G, vesicular stomatitis virus G protein.

with HIV-1 showed clear evidence of successful infection demonstrated by detection of episomal and integrated HIV-1 cDNA, and early gene expression in cells from spleen, thymus, and blood. Low-level plasma viremia was detectable in transgenic rats up to 7 wk after inoculation. These important steps provide a strong foundation for studies that will address the extent of HIV-1 replication, pathogenesis, and immune responses to HIV-1 in transgenic rats in vivo and elucidate their potential for testing of antiviral strategies.

Materials and Methods

Construction of Transgenic Rats. The transgenic vector for hCD4 has been described previously (32). The vector encoding hCCR5 was prepared to ensure expression in Th cells and in cells from the monocyte/macrophage lineage. For this purpose, an 8.4-kb BstBI-BamHI fragment from intron 1 of the human CD4 gene, shown to contain a monocyte-specific enhancer as well as the CD4 silencer (unpublished data), was inserted as a replacement for the ClaI–HindIII fragment in the middle of intron 1 in the murine construct "b," described previously (33). A 1.15-kb hCCR5 cDNA was inserted into the SalI site in exon 2, replacing the hCD2 cDNA in construct "b," thus yielding the plasmid pMΦE4A.CCR5 (Fig. 1 B). For preparation of transgenic rats, the plasmid vector sequences were excised by digestion with NotI. Rat founders for individual transgenic constructs were generated (Xenogen) by microinjection of the vector DNA into male pronuclei of fertilized oocytes from outbred Sprague-Dawley rats. Founders for the hCD4 transgene were identified by Southern blot analysis of EcoRV-digested tail DNA samples using a HindIII–BamHI fragment from pCD4neo (19) as a probe. Founders for hCCR5 were identified by PCR analysis from tail DNA samples using an internal primer set for hCCR5 cDNA (5′ primer: TCACTATGCTGCCGCCC. 3′ primer: AAACCAAAGTCCCACTGGGCG). Integration-positive founders were mated with nontransgenic Sprague-Dawley rats and F1 progeny were screened by flow cytometry for expression of human proteins in peripheral blood samples.

Antibodies and Flow Cytometry. FACS® analyses were performed as described previously (34), using FITC-, PE-, or APC-conjugated mAbs (BD PharMingen): anti-hCD4 (mAb Leu-3a); anti-hCCR5 (mAb 2D7); anti-rat (r)CD3 (mAb G4.18); anti-rCD4 (mAb OX-35); anti-rCD8a (mAb OX-8); anti-rat macrophage subset marker (ED2-like antigen, mAb HIS36); anti-rCD11b (mAb WT.5); anti-rCD11b/c (mAb OX-42); and anti-rCD45RA (mAb OX-33).

Immunohistochemistry. A standard three-step immunoperoxidase procedure using the Dako LSAB (R) 2 kit (Dako) was performed on formalin-fixed, paraffin-embedded tissues from PBS-perfused rats in principle as described previously (35). Tissues were sectioned onto silanized slides, allowed to dry, deparaffinized, and hydrated through graded ethanols. The tissues were pretreated with 3% H_2O_2 for 10 min to block endogenous peroxidases, heat-treated in citrate buffer, pH 6.0, for 10 min to retrieve antigens and finally buffered in PBS/casein. The slides were incubated with primary antibodies (anti-hCD4 mAb 1F6 [1:10]; Novacastra, or anti-hCCR5 mAb 3A9 [1:10]; BD PharMingen) for 2 h at room temperature, washed, and then stained after the kit protocol with 30-min incubations and final DAB reaction.

Primary Cells and Cultivation. Ex vivo cultures of primary rat lymphocytes, macrophages, or microglia, and cultures of PBMCs or human monocyte–derived macrophages were prepared and propagated as described previously (19).

Chemotaxis Assay. The migration of rCD4+-enriched primary rat splenocytes was determined by using a modification of the method described by Arai et al. (36) with Transwell® plates (pore size: 3 μm; diameter: 6.5 mm, Costar®; Corning, Inc.) and recombinant chemokines (R&D Systems).

Preparation of Viral Stocks. Molecular clones of pYU-2, pJR-CSF, and pNL4–3 were obtained from Beatrice Hahn (University of Alabama at Birmingham, Birmingham, AL), Irvin Chen (UCLA School of Medicine, Los Angeles, CA), and Malcom Martin (National Institutes of Health, Bethesda, MD), respectively, via the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. pYU-2b, a YU-2/HXB2 recombinant (37), was a gift from Warner Greene (Gladstone Institute of Virology and Immunology, San Francisco, CA). The molecular clone p49–5 (38) was a gift from Bruce Chesebro (Rocky Mountain Laboratories, Hami Hou, MT). pR7/3-YU-2-EGFP (39), which carries an EGFP gene within the *nef* locus driven by the 5′ LTR, was a gift from Mark Muesing (Aaron Diamond AIDS Research Center, New York, NY). Infectious virus stocks were prepared by transfecting 293T cells with proviral DNA as described previously (34). Viral stocks of JR-FL and Ada-M were obtained from Irvin Chen and Howard Gendelman (University of Nebraska Medical Center, Omaha, NE), respectively, via the NIH AIDS Research and Reference Reagent Program. The primary isolates C1 (40) and O3 (41) were gifts from James Mullins (University of Washington, Seattle, WA) and Ruth Connor (Aaron Diamond AIDS Research Center), respectively. Viral stocks were expanded by infection of heterologous human PBMCs. Infectious Ba-L stocks, which had been expanded on human monocyte-derived macrophages, were a gift from Teri Liegler (Gladstone Institute of Virology and Immunology).

For in vivo infections, YU-2 and R7/3-YU-2-EGFP stocks were concentrated using Centricon® Plus-80 columns (Millipore) following the manufacturer's protocol. The infectivity of viral stocks was determined by terminal dilution in quadruplicate on heterologous phytohémagglutinin-activated PBMCs. The titer of R7/3-YU-2-EGFP stocks was determined on HeLa hCD4/hCCR5 cells. The p24 CA concentration was assessed by ELISA (NEN Life Sciences). The molecular clone pNL-4–3 Luc E^-R^- (42), a replication-incompetent NL4–3 provirus (along with mutations in *env*, *nef*, and *vpr*), carrying a luciferase gene within the *nef* locus driven by the 5′ LTR, was a gift of Nathaniel Landau (Salk Institute for Biological Studies, La Jolla, CA) via the NIH AIDS Research and Reference Reagent Program. pVSV-G, the mammalian expression vector for VSV-G protein (43), was a gift from Jane Burns (University of California, San Diego, CA). The preparation of NL4–3 Luc E^-R^- pseudotype viruses with autologous or heterologous envelopes (Env) has been described previously (44).

Viral Infections. HIV-1 infections of ex vivo cultures were performed in 24-well (lymphocytes, macrophages) or 96-well plates (microglia, macrophages) with the indicated multiplicity of infection (MOI) or p24 CA concentrations. In infection studies on transgenic rat macrophages shown in Figs. 5 and 6, the following reagents were used: anti-hCCR5 mAb 2D7 (BD PharMingen) at 50 μg/ml; zidovudine (AZT) (3′-azido-3′deoxythymidine; Sigma-Aldrich) at 100 μM; Ritonavir (RTV) (Abbott Laboratories, Abbott Park, IL) at 1 μM; LPS (*Escherichia*

coli serotype 0128:B12; Sigma-Aldrich) at 100 ng/ml; or a formalin-fixed *Staphylococcus aureus* crude cell suspension (Sigma-Aldrich) at 0.01%. In vivo infections were performed by trained personnel in accordance with the guidelines and standards for humane animal experimentation set by the UCSF Committee on Research under an approved protocol. All rats were housed under SPF conditions with food and water ad libitum. For intravenous and intraperitoneal injections, rats were deeply anesthetized using isoflurane, and the tail vein or peritoneal cavity was cannulated with a 24G Abbocath®-T catheter (Abbott Laboratories) and YU-2 or R7/3-YU-2-GFP stocks were slowly infused (see specific Figure legends for viral titers). On the indicated days after inoculation, rats were killed with CO_2 and bilateral thoracotomy and organs were removed aseptically. Coded splenocyte, thymocyte, and PBMC samples were analyzed for the presence of HIV-1 cDNA products by PCR as described below. GFP-positive cells were detected by FACS® analyses. Serial blood samples were drawn from the jugular vein. HIV-1 vRNA assays of coded rat EDTA-treated plasma samples were performed by the Gladstone Core Virology Laboratory using the AMPLICOR HIV-1 MONITOR® test version 1.5 (gift from Roche Diagnostics).

Quantification of 2-LTR Circle Junctions. 2-LTR circle junctions were amplified by PCR from extracts of extrachromosomal DNA and quantified by Southern blotting as described previously (45).

Detection of Integrated Proviral DNA. A nested PCR strategy for the specific amplification of HIV-1 proviral DNA that is integrated into the rat genome was developed based on a previously reported method to evaluate HIV-1 integration close to human genomic *Alu* elements (46). We used a rodent identifier (ID) family consensus sequence that is highly abundant in the rat genome (47), to design the oligonucleotide primer BC1-A (5′-GGATTTAGCTCAGTGGTAGA-3′). Genomic DNA was extracted from formaldehyde-fixed cells using the DNAeasy® tissue kit (QIAGEN). During the first PCR reaction, cellular DNA was amplified with the HIV-1 LTR primer A (5′-AGGCAAGCTTTATTGAGGCTTAAGC-3′; reference 46) and primer BC1-A. *Taq* DNA polymerase (0.75 U/25 μl reaction; Stratagene), 200 μM of each dNTP and 500 nM of each primer were used. The reaction was run with the following program: (a) 3 min at 94°C, (b) 30 cycles of 30 s at 94°C, 30 s at 57°C, and 4 min at 72°C, and (c) 10 min at 72°C. A second nested PCR amplification, which allows amplification of a 357-basepair LTR fragment, was performed by using 1 μl of the first reaction with internal HIV-1 LTR primers NI-2 5′ and NI-2 3′ (46). This second PCR was performed using the same PCR conditions as for the first one, except that the annealing temperature was raised to 60°C, the extension time was 1 min, and amplification was run for 25 cycles. PCR products were analyzed by ethidium bromide/agarose gel electrophoresis.

Immunofluorescence Microscopy. Staining for intracellular p24 CA was performed on YU-2–infected rat microglia from hCD4/hCCR5-transgenic or hCD4-transgenic rats. Cells were washed with staining buffer (PBS containing 2% FBS) and then fixed in a 2% paraformaldehyde solution for 1 h on ice. After washing in staining buffer cells were permeabilized in 0.1% saponin for 15 min at room temperature. Cells were again washed in staining buffer and then incubated with a FITC-conjugated anti-p24 mAb (KC57, 1:10; Coulter Immunology) or a FITC-conjugated isotype control mAb. Subsequently, cells were stained with the Alexa Fluor® 488 Signal-Amplification kit (catalog no. A-11053; Molecular Probes) following the manufacturer's instructions. Images were acquired on an inverted Nikon TE-300 light microscope with SPOT RT digital camera using equal exposure times and identical digital filtering for all micrographs.

Results

Construction of Rats Transgenic for Human CD4 or Human CCR5. We developed several independent rat lines transgenic for either hCD4 or hCCR5. In humans, $CD4^+$ T lymphocytes, macrophages, and microglia constitute the

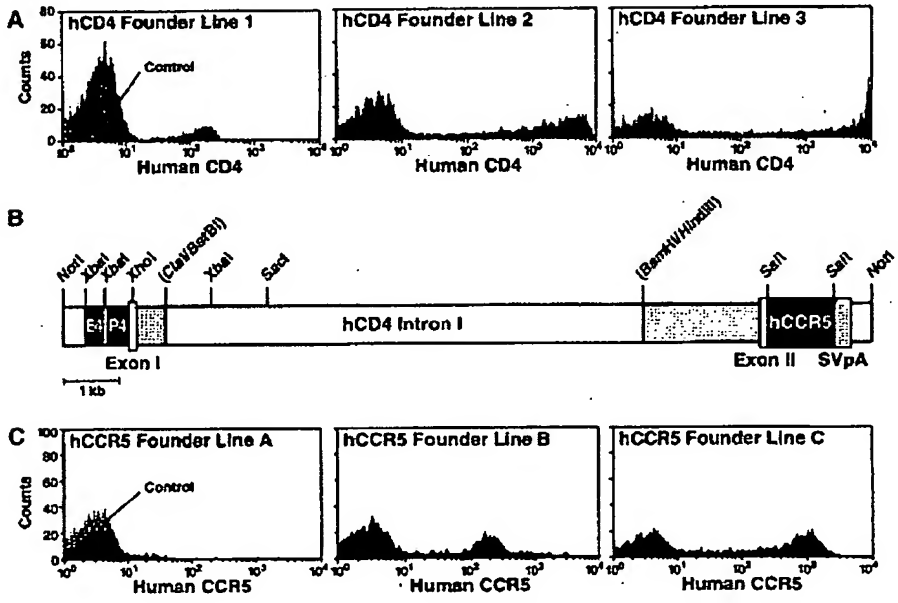

Figure 1. Transgenic rat lines expressing varying levels of hCD4 or hCCR5. Heparinized blood taken from (A) hCD4-transgenic rats, (C) hCCR5-transgenic rats, or nontransgenic littermate controls were stained with anti-hCD4 or anti-hCCR5 antibodies, respectively, and analyzed by flow cytometry. (B) Schematic representation of the transgenic vector for hCCR5 (pMΦE4A.CCR5; for details see Materials and Methods). E4/P4: murine *CD4* enhancer/promoter.

major reservoirs for HIV-1 replication. To mimic this pattern of susceptible tissues as closely as possible, we sought to target expression of the HIV-1 receptor complex to these specific cell types in transgenic rats. For expression of hCD4, we employed a construct, previously studied in transgenic mice (12, 32, 48), that contained the murine *CD4* enhancer linked to a 30-kb human *CD4* minigene that included the promoter and all of the exons (32). For expression of hCCR5, we used a chimeric mouse/human construct that directs expression of cDNA inserts in cells from the monocyte/macrophage lineage and Th cells (Fig. 1 B). Because human, but not murine, CD4 is expressed in the monocyte lineage, this construct was generated with the murine *CD4* enhancer and promoter plus a human *CD4* intron 1 sequence that we found to be required for expression in monocyte lineage cells, including macrophages, dendritic cells, and microglia (unpublished data). The human intronic sequence also contains the transcriptional silencer that restricts expression to $CD4^+$ T cells (33).

Rat founders for individual transgenic constructs were generated by microinjection of the vector DNA into male pronuclei of fertilized oocytes from outbred Sprague-Dawley rats. Five hCD4 integration founders and three hCCR5 integration founders were identified by Southern blot analysis or PCR analysis of tail DNA samples, respectively (data not shown).

As a first assessment of the expression of human transgenes in single-transgenic rats, peripheral blood lymphocytes from transgene-positive F1 progeny were stained with fluorochrome-conjugated antibodies specific for either hCD4 or hCCR5 and analyzed by flow cytometry. Three out of five hCD4 founder lines and all three hCCR5 founder lines expressed detectable levels of the respective human proteins on a subset of peripheral lymphocytes (Fig. 1 A and C). Each transgenic rat line had a unique expression level ranging from significant to very high, and these levels have proven to be stable and heritable. Several reports have demonstrated that surface levels of hCD4 or hCCR5 can be rate-limiting for HIV-1 entry into various cell types (49, 50). Consequently, in this study the transgenic lines with the highest expression levels of human transgenes, namely hCD4 founder line 3 (Fig. 1 A) and hCCR5 founder line C (Fig. 1 C), were interbred and used in subsequent experiments. Transgenic rats coexpressing hCD4 and hCCR5 were healthy and did not reveal any gross pathology compared with nontransgenic littermate controls in analyses of cellular blood composition and a head-to-tail necropsy (data not shown).

To characterize in more detail the cellular expression profiles of human transgenes in hCD4/hCCR5-transgenic rats, we first performed flow cytometry analyses with costaining for human transgenic proteins in conjunction with rat lineage– or lineage subset–specific markers. On peripheral lymphocytes from blood (Fig. 2 A, top panels) or spleen (data not shown), hCD4 and hCCR5 were coexpressed on the rat (r)$CD4^+$ subset, but not expressed on $rCD8^+$ T cells or B cells (data not shown). In thymocytes from transgenic rats both human transgenes were expressed

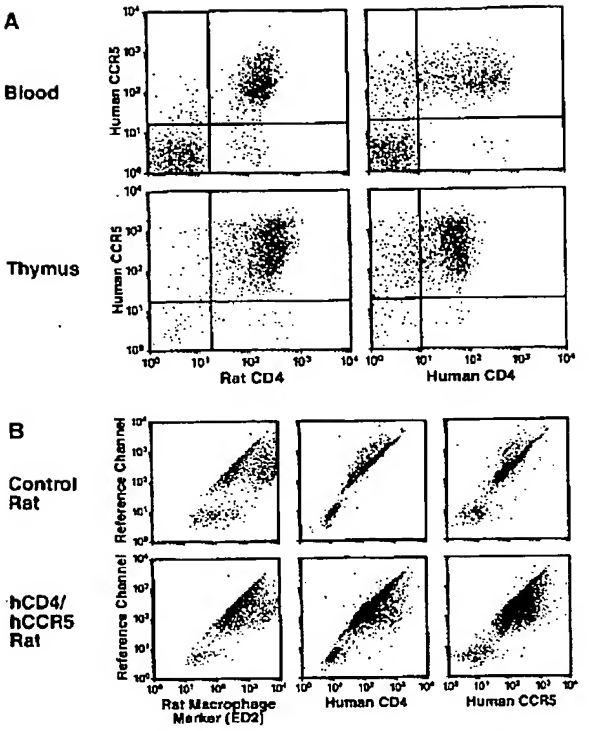

Figure 2. Cell type–restricted expression of hCD4 and hCCR5 in hCD4/hCCR5 transgenic rats. (A) Heparinized blood and thymocytes from a hCD4/hCCR5 transgenic rat were analyzed by flow cytometry for the expression of human transgenes and rCD4. Dot plots shown were gated on the total live lymphocyte population. (B) Subcultured primary rat spleen macrophages were stained for the expression of hCD4, hCCR5, or the rat macrophage activation marker rED2. Nontransgenic rat macrophages served as a control.

on $rCD4^+$ T cells (Fig. 2 A, bottom panels); both the $rCD4^+$ $rCD8^+$ subset, which constitutes the large majority of thymocytes, and the $rCD4^+$ $rCD8^-$ subset, expressed the transgenes (data not shown). Double-negative thymocytes ($rCD4^-$ $rCD8^-$ cells) had no detectable expression of either human transgene (data not shown). Expression analyses were also performed with cells from the monocyte/macrophage lineage isolated from these animals (Fig. 2 B). Since autofluorescence is much higher for these cells compared with lymphocytes, we used dot plots and two-parameter analyses in which unstained cells lie on the diagonal and positively stained cells are apparent due to their shift off the diagonal to the right. Using this approach, we found that macrophages isolated from nontransgenic control rats showed specific staining for the rat macrophage marker rED2, but not for hCD4 or hCCR5 (Fig. 2 B, top row). Importantly, macrophages from hCD4/hCCR5-transgenic rats expressed hCD4 and hCCR5 on the cell surface (Fig. 2 B, bottom row). Similarly, hCD4 and hCCR5 expression was detected on $rCD11b/c^+$ $rCD45^{low}$ rat microglia (51) cultured ex vivo (data not shown).

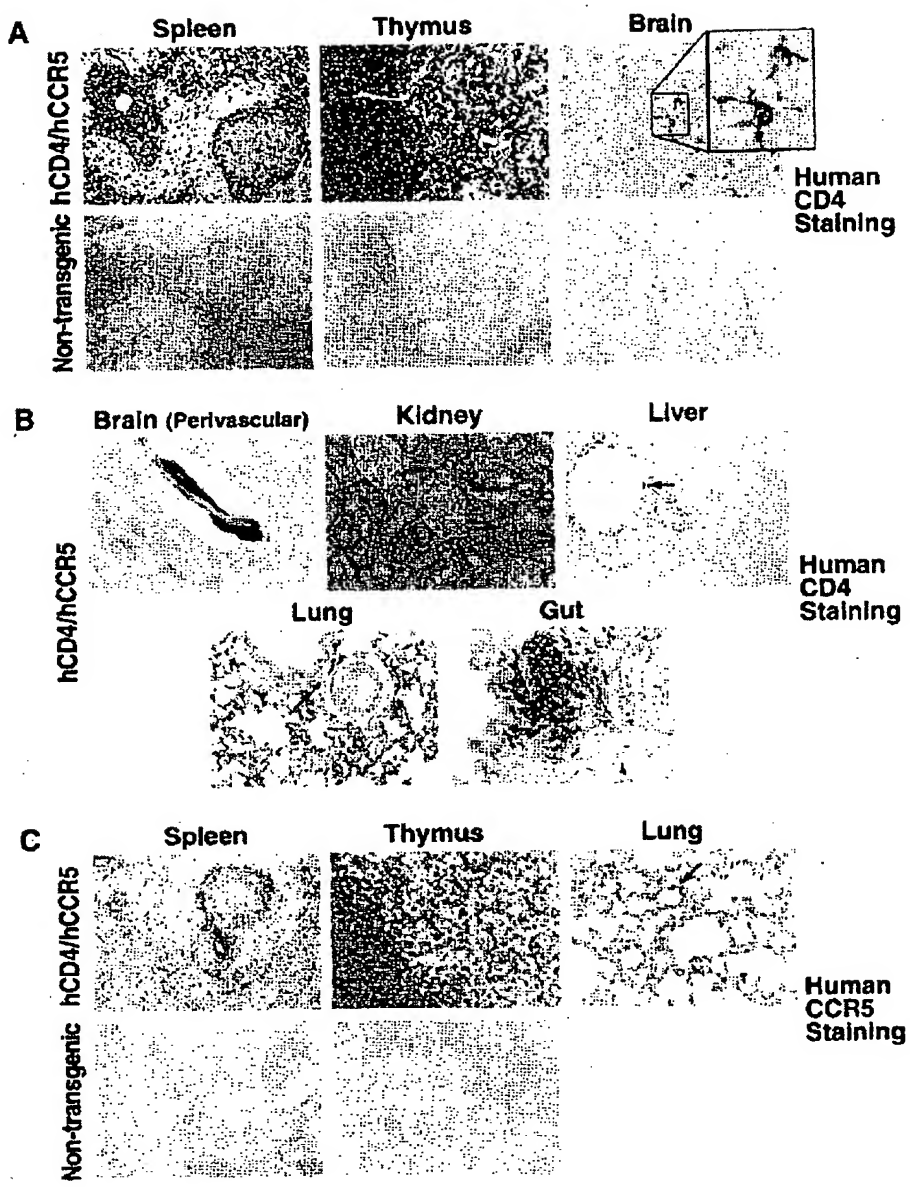

Figure 3. Selective expression of hCD4 and hCCR5 in various tissues from hCD4/hCCR5 transgenic rats. (A–C) Tissues were removed from saline-perfused rats and formalin fixed. Paraffin-embedded sections were stained with either (A and B) an anti-hCD4 antibody or (C) an anti-hCCR5 antibody and secondary step reagents. Equal exposure times were used for comparable micrographs from transgenic and nontransgenic sections. Original magnifications ranged from 40–400×.

Next, immunohistochemical stainings were performed as a complementary approach to assessing the expression of human transgenes, in particular providing insight into the tissue-specific distribution in situ. In transgenic rats, cells with the characteristic morphology and distribution patterns of T lymphocytes and macrophages expressed hCD4. Widespread, intense, and specific cellular hCD4 staining was detected in lymphocyte-rich spleen and thymus sections from a hCD4/hCCR5-transgenic rat (Fig. 3 A). Essentially no staining was detectable in tissues from a nontransgenic control rat with the anti-hCD4 staining (Fig. 3 A), nor did an isotype-matched negative control antibody yield a signal (data not shown). These results validate our immunohistochemistry methodology and confirm the results obtained by flow cytometry. We also conducted the same analyses with a number of other tissues from these animals. In brain sections, throughout the parenchyma we detected positive immunostaining in cells of microglial morphology with highly branched processes (Fig. 3 A). Similarly, larger cells with macrophage-like morphology and granular cytoplasm were frequently found in perivascular zones in the brain, most likely representing perivascu-

lar macrophages (Fig. 3 B). Expression of the hCD4 transgene was also found in tissue-resident macrophages in all other tissues analyzed including kidney, liver, and lung (Fig. 3 B). In the gut, submucosal lymphoid aggregates also showed abundant staining for hCD4. To define the expression pattern of hCCR5 in situ, parallel tissue sections from the same rats were stained with an antibody to this human chemokine receptor. Abundant expression of hCCR5 was found exclusively in the transgenic animal (Fig. 3 C); the expression pattern closely resembled that seen for hCD4, which was expected since both transgenic constructs share key regulatory elements. Collectively, these flow cytometry and immunohistochemistry data indicate that the expression of both human cell surface receptors had been successfully and exclusively targeted to the desired, biologically relevant cell types, that is, $CD4^+$ T cells, macrophages, and microglia in transgenic rats.

Rat Lymphocytes Expressing Human CCR5 Chemotax in Response to Human β–Chemokines. As a first assessment of the functional integrity of hCCR5 expressed on primary rat cells, $rCD4^+$ T lymphocytes from transgenic rats were tested for in vitro migration toward active human chemokines at varying concentrations. Lymphocytes from transgenic rats expressing hCD4 and hCCR5, but not hCD4 alone, chemotaxed in response to the natural hCCR5 β–chemokine ligands human regulated upon activation, normal T cell expressed and secreted (RANTES) and human macrophage inflammatory protein (MIP)-1α (Fig. 4). Similar results were obtained with hMIP-1β (data not shown). The chemotaxis of hCCR5-expressing lymphocytes followed a typical biphasic response to increasing concentrations of human β–chemokines (Fig. 4 A). Similar results were obtained with lymphocytes expressing hCCR5 in the absence of hCD4 (data not shown). Comparable chemotaxis of lymphocyte preparations from transgenic and nontransgenic rats toward the CXCR4 ligand stromal cell–derived factor 1 confirmed cellular viability and the validity of the assay. These results demonstrate that hCCR5 in primary transgenic rat cells retained chemokine-mediated signaling functions linked to the proper biologic responses, suggesting appropriate expression, post-translational modification, and subcellular localization of this human seven transmembrane G-protein–coupled receptor in this species context.

Primary Macrophages and Microglia from Transgenic Rats Can Be Productively Infected by Recombinant and Primary Strains of HIV-1. To assess whether expression of the HIV-1 receptor complex on primary rat cells rendered them permissive for viral infection, transgenic macrophages were first challenged with a set of HIV-1 luciferase reporter viruses. These pseudotypes are based on an Env-deficient HIV-1 proviral backbone containing the firefly luciferase gene within the nef gene locus (pNL4–3 Luc E^-R^-; reference 42), expression of which provides a quantitative marker of successful entry, reverse transcription, integration, and early viral gene expression in a given target cell. Primary macrophages from double-transgenic rats showed coreceptor-specific entry and high-level, early HIV-1 gene expression in ex vivo cultures (Fig. 5 A). Macrophages from hCD4/hCCR5-transgenic rats were highly permissive for infection by the R5 Env pseudotypes JR-FL and Ada-M, but nonpermissive for the hCXCR4-using (X4) Env pseudotype NL4–3. As expected, all macrophage cultures were permissive for the VSV-G pseudotype, which confers entry into a wide range of mammalian cells. As an additional control of specificity, pretreatment of hCD4/hCCR5-expressing rat macrophages with an anti-hCCR5 antibody reduced signals for the HIV-1 JR-FL Env pseudotype by 94%, but did not significantly affect the infection by the VSV-G pseudotype (data not shown). The low-level susceptibility of hCD4 single-transgenic rat macrophages for R5 pseudotypes may indicate the utilization of an endogenous rat cell surface molecule as an inefficient coreceptor for HIV-1 entry.

Next, transgenic rat macrophages were challenged with different, replication-competent strains of HIV-1. Primary macrophages from hCD4/hCCR5-transgenic rats could be productively infected by R5 viruses (Fig. 5 B–D). In particular, double-transgenic rat macrophages challenged with the recombinant R5 strain YU-2b (37) showed increasing p24 CA concentrations over the course of 10 d and reaching up to 18 ng per ml (Fig. 5 B). In contrast, rat macro-

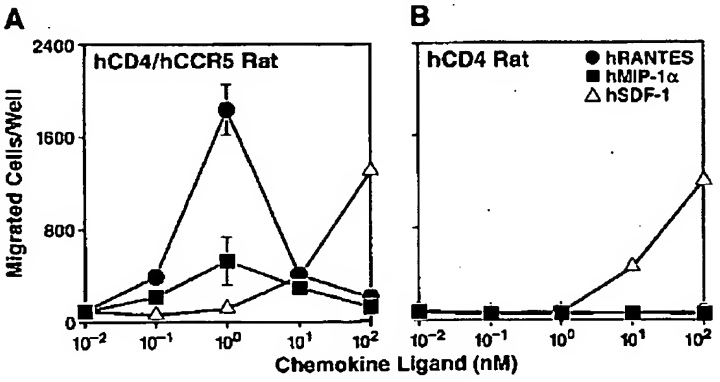

Figure 4. β-chemokine–mediated chemotaxis of hCCR5-expressing, primary rat T lymphocytes. Single cell suspensions of spleen from (A) a hCD4/hCCR5-transgenic rat, and (B) a hCD4-transgenic rat, were enriched for $rCD4^+$ cells by antibody-mediated depletion of $rCD11b^+$, $rCD45R^+$, and $rCD8^+$ mononuclear cells. Subsequently, a transwell chemotaxis assay was performed using human β-chemokines hRANTES and hMIP-1α as specific ligands. human stromal cell-derived factor 1 served as a positive control for viability. The relative number of migrated cells per well was determined and is presented as the arithmetic mean ± SD of triplicates. Results are representative of two independent experiments.

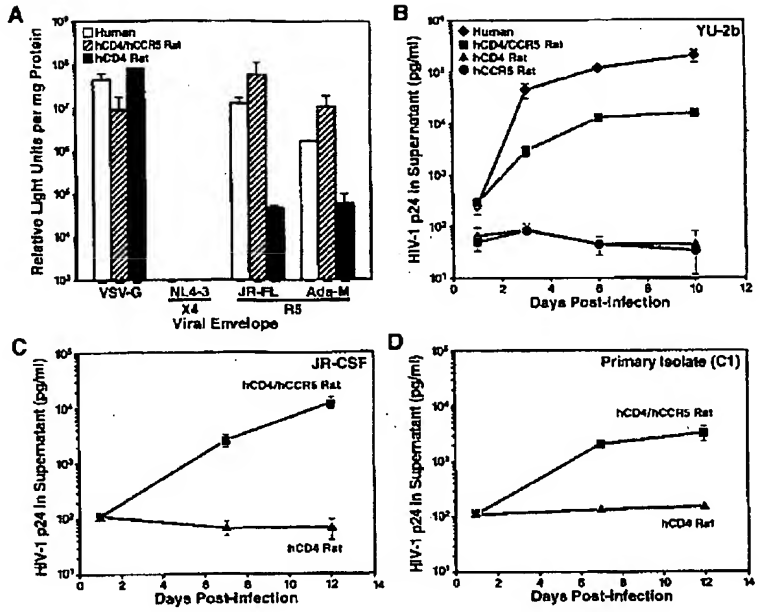

Figure 5. Primary rat macrophages coexpressing hCD4 and hCCR5 are productively infected by recombinant and primary strains of HIV-1. (A) Thymus-derived rat macrophages from a hCD4/hCCR5 transgenic rat, a hCD4 transgenic rat, or CD14$^+$ human monocyte–derived macrophages were cultured in 24-well plates and infected with the indicated HIV-1 luciferase reporter pseudotypes. The luciferase activity in cellular lysates was determined 4 d after infection and is presented here as "relative light units" per milligrams of protein. (B–D) Spleen-derived macrophages from hCD4/hCCR5 transgenic rats or single-transgenic rats (hCD4 or hCCR5 single-positive, as indicated), or human monocyte–derived macrophages, were infected with stocks of replication-competent HIV-1 YU-2b, JR-CSF, or the primary isolate C1 (20 ng p24 CA per well) and p24 CA levels in the supernatant were measured at the indicated time points. All values shown represent arithmetic means of triplicates ± SD.

phages single-transgenic for either hCD4 or hCCR5 were not productively infected, as revealed by p24 CA concentrations at background level. The parallel infection of human monocyte-derived macrophages yielded 9–15-fold higher p24 CA levels. Importantly, double-transgenic rat macrophages could be productively infected by a variety of R5 viruses, including JR-CSF (Fig. 5 C), cloned from the cerebrospinal fluid of an AIDS patient (52), the primary, patient-derived isolates C1 (40) (Fig. 5 D) and O3 (41) (data not shown), as well as the laboratory-adapted, macrophage-tropic strain Ada-M (53) and the NL4–3-based molecular clone 49.5 carrying the V3 loop from Ba-L (38) (data not shown).

Next, to investigate further whether these infections were productive, we studied the effect of a reverse transcriptase or protease inhibitor on HIV-1 infection in macrophages from hCD4/hCCR5-transgenic rats. The reverse transcriptase inhibitor AZT completely abrogated infection by YU-2 (Fig. 6 A), demonstrating that the p24 CA signals depend upon completion of this essential early enzymatic reaction in the viral life cycle and cannot be accounted for by input virus. The processing of the HIV-1 polyproteins p55 Gag and p160 Gag-Pol by the virally encoded protease into functional subunits is an essential step for HIV-1 maturation. Treatment with a protease inhibitor renders newly synthesized virus particles noninfectious (54). In cultures of hCD4/hCCR5-transgenic macrophages infected with YU-2, the presence of the protease inhibitor RTV significantly reduced the secreted p24 CA concentration compared with untreated controls (Fig. 6 A). Based on these considerations, the infection in double-transgenic macrophage cultures likely represents a spreading infection: First, transfer of cell-free supernatant from an infected primary hCD4/hCCR5-transgenic macrophage culture at 5 d after infection onto a second culture from the same rat led to a productive infection in the recipient culture (Fig. 6 C), albeit at low levels. Second, infection of hCD4/hCCR5-transgenic macrophage cultures with YU-2 at a very "low" MOI (0.05) resulted in comparable p24 peak concentrations to that achieved in a "high" MOI (0.5) infection, but with a delay of ~8 d for the cultures infected at the "low" MOI (data not shown).

In addition, certain bacterial compounds, including endotoxins like LPS, have under certain conditions been shown to enhance HIV-1 replication in human cells and macrophages from HIV-1 provirus-transgenic mice ex vivo (55, 56). In hCD4/hCCR5-expressing rat macrophages, both LPS and a formalin-fixed *Staphylococcus aureus* cell suspension measurably enhanced HIV-1 replication (Fig. 6 B). Collectively, our data indicate that a variety of HIV-1 isolates can efficiently and productively infect primary macrophages from hCD4/hCCR5-transgenic rats in a coreceptor-dependent fashion. Furthermore, antiviral drugs that target specific steps in the viral life cycle inhibit HIV-1 replication in primary ex vivo cultures from transgenic rats.

After these provocative results with macrophages, we also investigated the permissivity of brain-derived microglia from transgenic rats. Primary microglia were isolated from neonatal rat brains, enriched by subcultivation, and characterized as described previously (19). Microglial cultures revealed expected morphologic characteristics, including ruffled edges with occasional long, branched processes, small and heterochromatic nuclei, granular vesicles within the cytoplasm, as well as an absence of staining for the astrocyte-specific glial fibrillary acidic protein marker (data not shown). First, to assess the integrity of the early phase of the

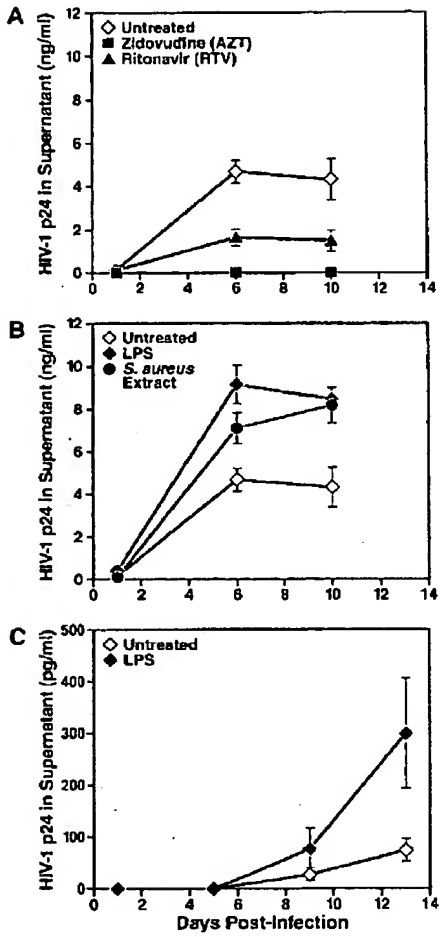

Figure 6. Effect of antiretroviral drugs and bacterial components on HIV-1 replication in transgenic rat macrophages. Spleen-derived macrophage cultures from hCD4/hCCR5 transgenic rats were pretreated with (A) AZT overnight, or RTV, (B) LPS or *Staphylococcus aureus* extract for 5 min before challenge with YU-2 (MOI 0.3). Cells were extensively washed the next day and then continuously cultivated for 10 d in the presence of the respective reagents. (C) Transfer of cell-free supernatants from YU-2-infected macrophages from a hCD4/hCCR5 transgenic rat 5 d after infection onto uninfected cultures from the same rat (p24 CA transferred: 420 pg [untreated], 1160 pg [LPS]). After overnight infection and subsequent washing, the supernatant of secondary cultures was monitored serially for p24 CA concentrations. All p24 CA values shown are the arithmetic means of triplicates ± SD.

HIV-1 replication cycle, microglia were challenged with a series of HIV-1 luciferase reporter viruses. As seen with macrophages from hCD4/hCCR5-transgenic rats, double-transgenic microglia also supported coreceptor-specific entry and robust early HIV-1 gene expression (Fig. 7 A). Comparable results were obtained also for the luciferase reporter pseudotype containing the Env from YU-2 or JR-CSF (data not shown). In contrast, microglia from a non-transgenic control rat were not permissive for either the R5 or X4 pseudotypes, but were readily infected by the VSV-G pseudotype. Second, we sought to determine whether transgenic expression of human entry cofactors on rat microglia would also confer permissivity for the full HIV-1 replication cycle. Microglial cultures from a hCD4/hCCR5-transgenic and a hCD4-transgenic rat were challenged overnight with the R5 virus YU-2 or VSV-G pseudotyped NL4–3 (NL4–3/(VSV-G)). The following day, cultures were extensively washed and 6 d after infection the p24 CA concentration in culture supernatants was determined. In addition, microglia were subsequently fixed and processed for intracellular p24 CA staining. Microglial cultures from double-transgenic rats, but not nontransgenic rats, showed clear evidence for productive infection as indicated both by significant levels of secreted p24 CA (Fig. 7 B) and by the abundant presence of intracellular viral capsid antigen (Fig. 7 C). Interestingly, we occasionally observed large, syncytia-like, multinucleated cellular aggregates in these infected cultures, but not in uninfected controls (data not shown). In contrast, microglia from hCD4 single-transgenic control rats secreted significant p24 CA concentrations after infection with NL4–3/(VSV-G), but not after challenge with the R5 virus (Fig. 7 B). These results demonstrate that coexpression of hCD4 and hCCR5 on microglia from transgenic rats confers coreceptor-specific and substantial infection by R5 strains of HIV-1.

Coexpression of Human CD4 and Human CCR5 Transgenes Overcomes the Entry Block in Primary Rat Lymphocytes, but Does Not Render Them Permissive for Productive HIV-1 Infection. We next sought to determine if coexpression of human transgenes on lymphocytes was also sufficient to overcome the entry block to HIV-1 infection. Mitogen/IL-2–activated primary lymphocytes from hCD4/hCCR5-transgenic rats showed robust signals after infections with the R5 Env pseudotypes Ba-L, Ada-M, or JR-FL (Fig. 8 A). Luciferase levels in transgenic rat lymphocytes were only three to sevenfold lower than those seen in parallel infections of activated primary human T lymphocytes. As a control of specificity, double-transgenic rat lymphocytes were demonstrated to be nonpermissive to infection by the X4 Env pseudotype NL4–3, and nontransgenic control lymphocytes were nonpermissive for either the R5 Env or the X4 Env pseudotypes. These results demonstrate that expression of the HIV-1 receptor complex was sufficient to confer coreceptor-specific entry and robust early, Rev-independent HIV-1 gene expression in primary $CD4^+$ T lymphocytes from transgenic rats.

Challenging the same set of lymphocyte cultures with the replication-competent R5 virus YU-2b revealed that hCD4/hCCR5-transgenic lymphocytes did not secrete significant concentrations of p24 CA (Fig. 8 B), despite clear signs of progression through the first part of the replication cycle, including the abundant expression of a Nef/luciferase reporter (Fig. 8 A). This nonpermissive phenotype was also not overcome by other means of activating rat T lymphocytes, including rCD3/rCD28 costimulation (data not shown). Similar results were obtained with infections by the R5 virus Ba-L (data not shown). In contrast,

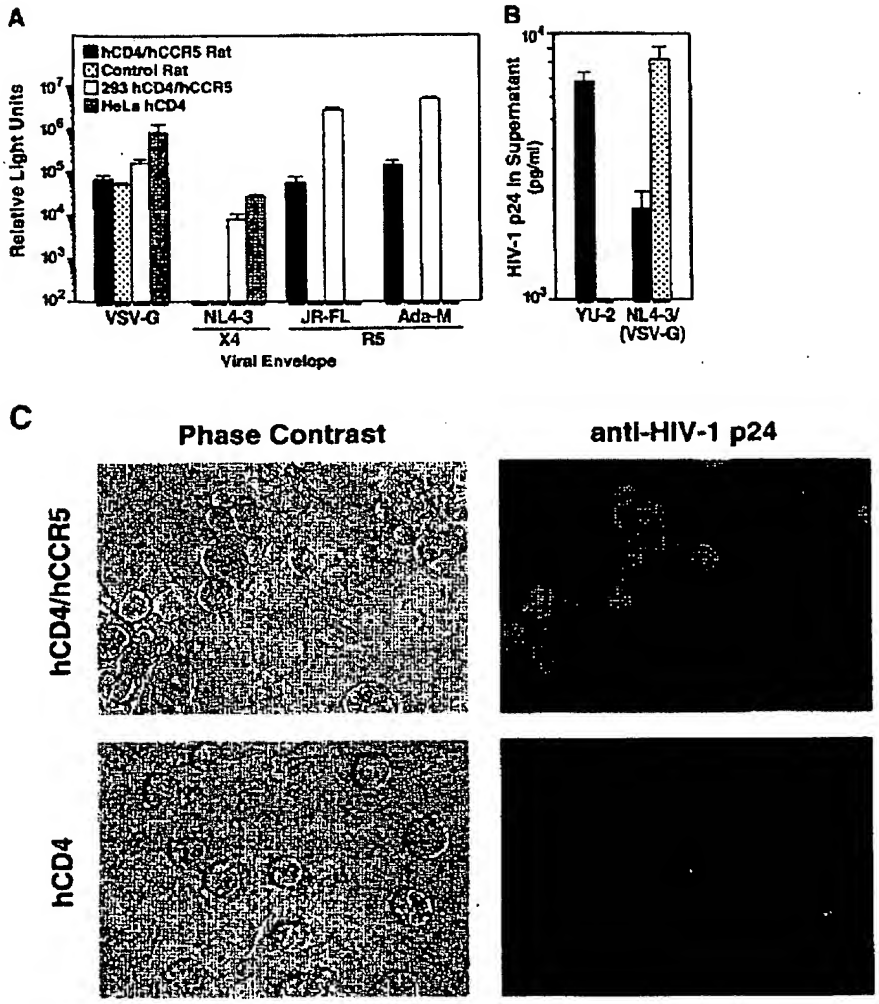

Figure 7. Primary microglia from hCD4/hCCR5 transgenic rats are permissive for productive infection by R5 viruses. (A) Primary microglia from hCD4/hCCR5 transgenic rats or nontransgenic control rats cultured in 96-well plates were challenged with the indicated pseudotyped HIV-1 luciferase reporter viruses and luciferase activity in cellular lysates was determined 2 d after infection. Human 293 hCD4/hCCR5 and HeLa hCD4 cells served as controls. (B) Microglial cultures from hCD4/hCCR5 transgenic or hCD4 transgenic control rats were infected with replication-competent YU-2 (1.5 ng p24 CA per well) or VSV-G pseudotyped NL4–3 (NL4–3/[VSV-G]) (0.7 ng p24 CA per well) overnight and washed extensively the next day, with a postwash p24 CA concentration of <300 pg/ml for all cultures. 6 d after infection the p24 CA concentration in supernatants was quantified and is represented as arithmetic means ± SEM of triplicates. (C) Infected microglia from (B) were fixed, permeabilized, and processed for intracellular p24 CA immunocytochemistry. Phase contrasts are shown on the left, and staining for p24 CA is shown on the right. Staining with an isotype control antibody yielded negligible background signals (data not shown).

human lymphocytes showed a typical infection kinetic over the course of 10 d. Thus, cells from the monocyte-macrophage lineage, but not lymphocytes, from double-transgenic rats could be productively infected by R5 strains of HIV-1.

Transgenic Rats Can Be Infected With HIV-1 In Vivo. Finally, we sought to address whether transgenic rats could be infected with HIV-1 in vivo. In a first experiment, hCD4/hCCR5-transgenic and nontransgenic control rats (n = 4 per group) were challenged intravenously with YU-2. At day 3 or 16 after the systemic challenge two rats from each group were killed, and spleens were removed and analyzed for signs of HIV-1 infection. We used a recently described PCR approach to detect circularized HIV-1 cDNA genomes containing two long-terminal repeats (2-LTR circles) (45). 2-LTR circles are formed from linear full-length cDNAs that do not integrate into the host genome, but circularize to form episomes. Their presence in a cell is an established surrogate for successful cellular entry, reverse transcription, and nuclear import of HIV-1. Advantages of this analysis include its unambiguous discrimination from the genomic RNA of the viral inoculum and its quantitative nature. In all transgenic rats, 2-LTR circles were readily detectable in splenocyte samples (Fig. 9), both on day 3 (rat no. 1: 1128 copies per 10^8 splenocytes; rat no. 2: 456 copies per 10^8 splenocytes) and day 16 (rat no. 3: 608 copies per 10^8 splenocytes; rat no. 4: 144 copies per 10^8 splenocytes) after challenge. In contrast, in none of the splenocyte samples from the four nontransgenic rats could 2-LTR circle signals be amplified.

In a second in vivo experiment, we sought to detect proviral DNA that had integrated into the genome of rat cells following systemic viral challenge. Based on a previously reported nested PCR strategy to specifically amplify HIV-1 integrated close to genomic human Alu repeat elements (46) we developed a similar PCR assay using a

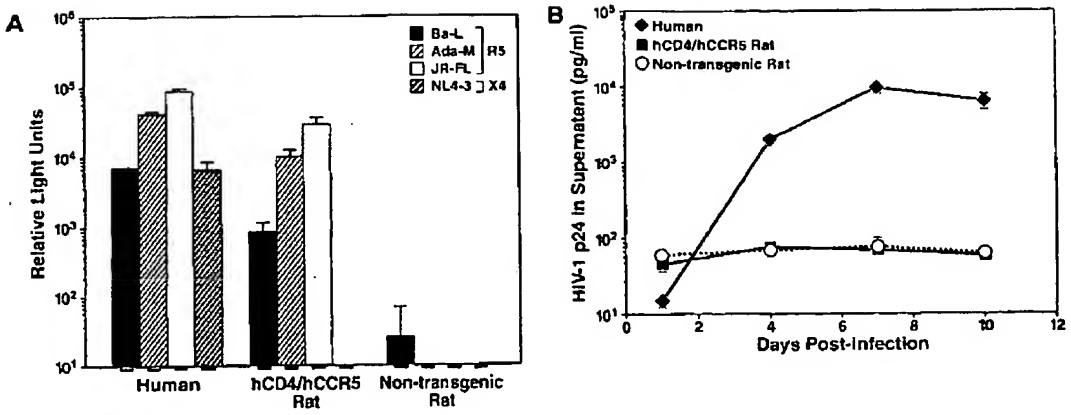

Figure 8. Coexpression of hCD4 and hCCR5 transgenes renders primary rat T lymphocytes permissive for entry and early gene expression of R5 viruses, but does not allow for a productive infection. (A) Mitogen/IL-2–activated, spleen-derived lymphocytes from a hCD4/hCCR5-transgenic rat, a nontransgenic littermate control rat or human lymphocytes were infected with the indicated pseudotyped HIV-1 luciferase reporter viruses. Luciferase activity in cellular lysates was determined 4 d after infection. (B) In parallel, the same lymphocyte cultures were infected with the replication-competent R5 strain YU-2b. The HIV-1 p24 CA concentration in culture supernatants was determined at the indicated time points. All values represent the arithmetic means ± SD of triplicates.

primer specific for a rodent ID family consensus sequence, that is highly redundant in the rat genome (47) (see Materials and Methods for details). Integrated proviral DNA was amplified from genomic DNA samples derived from spleen samples of a hCD4/hCCR5-transgenic rat (Tg #X) infected with YU-2 in vivo (Fig. 9 B, lane 7) or from ex vivo–infected thymocytes as a control (data not shown). The specific 357 bp product was not amplified from uninfected splenocyte samples, splenocyte samples from an infected nontransgenic rat, or thymus samples from Tg #X (Fig. 9 B, lanes 2–6). Similarly, omission of the ID repeat primer from the first PCR for DNA samples from spleen of Tg #X resulted in a loss of the specific product following the second PCR (Fig. 9 B, compare lanes 6 and 7), demonstrating that the exponential amplification of integrated HIV-1 cDNA in the first PCR reaction was necessary for the generation of the LTR-specific product and that extrachromosomal viral DNA cannot account for the band observed.

In a third experiment, we sought to visualize and quantify HIV-1 gene expression in vivo directly. Rats were challenged intravenously with the recombinant R5 virus R7/3-YU-2-EGFP (39) which carries an EGFP gene within the *nef* locus driven by the 5′ LTR. Lymphocytes and macrophages from spleen (Fig. 10) and PBMCs (data not shown) from both hCD4/hCCR5-transgenic rats, but

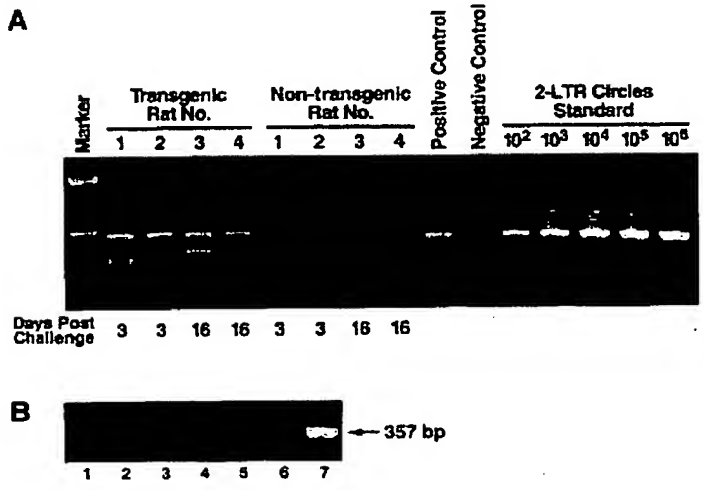

Figure 9. Detection of episomal and integrated HIV-1 cDNA in splenocytes from transgenic, but not nontransgenic rats, challenged with HIV-1 in vivo. (A) hCD4/hCCR5 transgenic and nontransgenic control rats (n = 4 per group) were challenged with YU-2 (8×10^5 $TCID_{50}$ per rat), intravenously. On either day 3 or 16 after challenge two rats from each group were killed and spleens removed. Nuclear extrachromosomal DNA was isolated from splenocytes and analyzed for the presence of 2-LTR circles by PCR as described recently (reference 45). Shown is an ethidium bromide-stained gel of PCR products. Positive control: Ex vivo–infected hCD4/hCCR5 transgenic splenocytes; negative control: no DNA. (B) A hCD4/hCCR5-transgenic (Tg #X) and a nontransgenic rat (NTg) were infected with YU-2 (5×10^7 $TCID_{50}$ per rat) intravenously and spleen and thymus were harvested on day 5 after challenge. Integrated proviral DNA in genomic extracts was detected by nested PCR (see Materials and Methods for details). Lanes: (1) no template; (2) NTg spleen; (3) Uninfected Tg #Y spleen; (4) Tg #X thymus, no ID primer; (5) Tg #X thymus; (6) Tg #X spleen, no ID primer; (7) Tg #X spleen. Except for lanes 4 and 6 both ID primer BC1-A and LTR primer A were added to the first PCR reaction. Shown is an ethidium bromide-stained gel of the expected 357 bp PCR product.

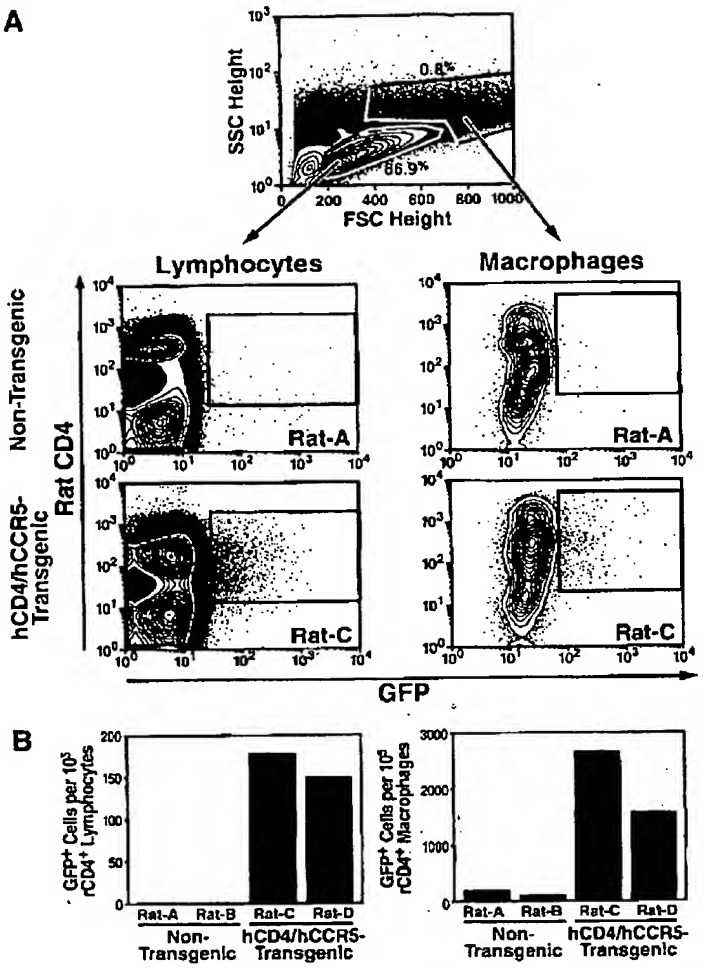

Figure 10. Early HIV-1 gene expression in splenocytes from hCD4/hCCR5 transgenic rats after intravenous infection with R7/3-YU-2-EGFP. Nontransgenic (rat A and B) and double-transgenic rats (rat C and D) were challenged with R7/3-YU-2-EGFP (9×10^6 $TCID_{50}$ per rat). On day 4 after infection, spleen and PBMC samples were harvested and analyzed by multicolor FACS® analysis. (A) Splenocytes were gated on "lymphocyte" and "macrophage" populations based on FSC/SSC profiles previously defined by backgating of $rCD3^+$ and $rCD11b/c^+$ cells, respectively (data not shown). Viable, 7-AAD$^-$ cells in the respective gates are presented as dot plots showing GFP expression relative to rCD4(APC) expression. (B) Quantitative analysis of GFP^+ $rCD4^-$ splenocyte subsets. Data on $\sim 4 \times 10^6$ cells were acquired for each sample.

not from the infected nontransgenic rats or an uninfected transgenic rat, contained a significant population of GFP^+ cells (Fig. 10, and data not shown). A further indication of specificity was that the overwhelming majority of GFP^+ cells were $rCD4^-$ cells (Fig. 10 A), which are the only cells that in these animals express the HIV-1 receptor complex. The frequency of GFP^+rCD4^+ splenocytes was ~0.15% for lymphocytes and 2% for macrophages in transgenic rats (Fig. 10 B). In peripheral blood the frequency of GFP^+rCD4^+ lymphocytes was ~0.01%, with a 10-fold lower background frequency in nontransgenic rats (data not shown).

In a fourth experiment, hCD4/hCCR5-transgenic and nontransgenic control rats ($n = 6$ per group) were challenged with YU-2 by intraperitoneal inoculation. Also, a group of mock-infected double-transgenic control rats was included in this study. Control rats exhibited no gross clinical abnormalities through the postinoculation observation period. Unexpectedly, one HIV-1-inoculated transgenic rat (#521) was observed to have a generalized tonic-clonic seizure 21 d after infection, from which it recovered spontaneously. This transgenic animal was found dead at 43 d after infection, but pathologic assessment was obscured by severe postmortem tissue degradation during the time interval from death to discovery.

Plasma viral loads were monitored in all of these rats weekly for 7 wk after inoculation, and again at three subsequent time points (weeks 11, 13, and 25 after inoculation) (Fig. 11). All samples from the nontransgenic control animals and mock-infected double-transgenic rats (data not shown) were negative (<20 HIV-1 RNA copies per milliliter), indicating the specificity of the experimental system. In contrast, at least one plasma sample each from five of six inoculated hCD4/hCCR5-transgenic rats was positive (range: 20–151 HIV-1 RNA copies per milliliter), while three of them had positive samples at two separate time points. The latest time point for which plasma viremia was detected was 7 wk after inoculation

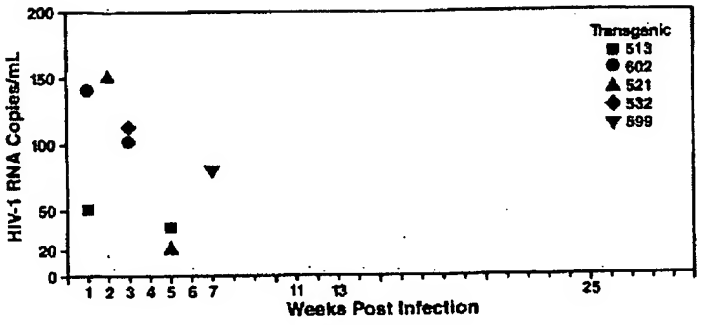

Figure 11. Plasma viremia in hCD4/hCCR5 transgenic rats after intraperitoneal inoculation with YU-2. Six double-transgenic and six nontransgenic rats were inoculated intraperitoneally with YU-2 (10^7 $TCID_{50}$ per rat). Plasma sampling was performed at each of the time points identified with a bold number on the x-axis (weeks 1, 2, 3, 4, 5, 6, 7, 11, 13, and 25 after infection) and HIV-1 vRNA concentrations were determined by the ultrasensitive Roche Amplicor HIV-1 Monitor tests with a limit of detection of 20 HIV-1 RNA copies per milliliter. Shown in this figure are all plasma samples with detectable viremia (transgenic rats 513, 602, 521, 532, and 599). None of the samples from the nontransgenic rats (rats 530, 568, 603, 655, 706, and 523) or one of six transgenic rats (rat 518) scored positive in this analysis.

(#599). At 6 mo after inoculation, two rats from each group were killed (hCD4/hCCR5-transgenic, HIV-1-infected: #513, #532; nontransgenic, HIV-1 infected: #530, #568; hCD4/hCCR5-transgenic, mock-infected: #584, #615) and spleen and thymus samples were analyzed for the presence of 2-LTR circles. Only the tissues from HIV-1 infected, transgenic rats (#513 (spleen) and #532 (spleen and thymus) contained low, but detectable numbers of 2-LTR circles, ranging from 36–108 copies per organ.

Discussion

The development of a transgenic small animal model permissive for HIV-1 infection would aid the study of viral transmission, pathogenesis, and the testing of therapeutic strategies including vaccines. A major obstacle to this end has been the inability of cells from present transgenic rabbit or mouse models to support a robust productive HIV-1 infection (11, 12, 57). We have previously shown that cellular entry constitutes the only absolute block to HIV-1 replication in certain rat cell lines, and that this restriction can be overcome by coexpression of hCD4 and hCCR5 (19). Studies with pseudotyped HIV-1 revealed that primary cells from the monocyte/macrophage lineage from various rat strains supported all steps in the viral replication cycle, including the production of infectious HIV-1. Based on these findings, we hypothesized that reconstitution of the HIV-1 receptor complex in rats would overcome the entry block in primary cells and recapitulate the full viral replication cycle in at least some cell types such as macrophages. Specifically, the mouse CD4 enhancer and the human CD4 intron 1, which contains a monocyte lineage-specific enhancer (unpublished data), were included in both transgenes, as was the human intronic silencer that shuts off CD4 expression in CD8 lineage T cells. A definitive murine promoter element for CCR5 has not yet been identified, and this strategy assured coexpression of hCD4 and hCCR5, which is an absolute requirement for efficient cellular entry of R5 viruses. Several independent, single-transgenic rat lines were generated that displayed distinct expression levels of human transgenes. Detailed expression analyses using flow cytometry and immunohistochemistry indicated that both hCD4 and hCCR5 had been successfully and exclusively targeted to the desired, biologically relevant cell types in transgenic rats.

Although hCCR5-mediated signaling is not required for HIV-1 infection (34, 58, 59), coreceptor-mediated signaling events induced by R5 Env have been suggested to be contributory to HIV-1–related pathology (60, 61). We thus sought to determine whether or not hCCR5 expressed on primary cells from transgenic rats preserved its competence as a signal-transducing chemokine receptor. We found that hCCR5-expressing lymphocytes chemotaxed in response to the natural human ligands for hCCR5, the β-chemokines hRANTES, hMIP-1α, and hMIP-1β, demonstrating that hCCR5 in primary transgenic rat cells retained signaling functions linked to the proper biologic responses. In this context it is also noteworthy that transgenic rats expressing high levels of hCCR5 were healthy and did not reveal any gross abnormalities, particularly within the hematopoietic system. This observation is consistent with the absence of a known physiological phenotype in humans that express markedly different levels of hCCR5 (30).

Coexpression of hCD4 and hCCR5 in transgenic rats indeed rendered primary rat cells susceptible to entry of HIV-1. This process was both hCD4-dependent and coreceptor-specific as demonstrated by several lines of experimental evidence. First, single-transgenic or nontransgenic rat cells were largely nonpermissive. Second, double-transgenic cells were permissive selectively for R5, but not X4, viruses. Third, an anti-hCCR5 antibody specifically blocked infection by an R5 virus. Moreover, entry was not restricted to specific R5 Env, since diverse R5 strains tested were capable of infecting hCD4/hCCR5-expressing primary rat cells. These results suggest that hCD4 and hCCR5 expressed in transgenic rats have the required posttranslational modifications and appropriate subcellular localization to function as an efficient HIV-1 receptor complex in this species context.

Consistent with our earlier studies on nontransgenic rat-derived cells (19), we found that primary cells from the monocyte/macrophage lineage from hCD4/hCCR5-transgenic rats allowed for a productive infection by R5 viruses in ex vivo cultures. Remarkably, levels of productive infection were one to two orders of magnitude higher than those described for comparable transgenic mouse models

(11, 12). R5 primary isolates, molecular clones, and recombinant strains of HIV-1 were all capable of productively infecting hCD4/hCCR5 transgenic rat macrophages. Furthermore, the significant inhibitory effect of a reverse transcriptase and a protease inhibitor further confirmed the productive nature of viral replication in these transgenic rat macrophage cultures. Evidence from low MOI inoculations and from supernatant transfer experiments suggests that the infection in these ex vivo macrophage cultures was spreading. This fact is consistent with our earlier observation that virus released by primary rat macrophages is highly infectious (19). These findings are also important to provide context for the interpretation of viral dynamics in vivo.

In humans, macrophages and microglia are important targets of HIV-1 that support virus replication in vivo (62–66). Several studies have implicated macrophages as a long-term reservoir for HIV-1 in infected individuals (67, 68) and macrophages have been suggested as a source of increasing viremia in later stages of HIV disease, particularly during opportunistic infections (69). Similarly, in a Rhesus macaque study, macrophages were implicated as the principal reservoir for sustained high virus load after the depletion of $CD4^+$ T cells by a simian immunodeficiency virus (SIV)/HIV-1 chimera (70). Regarding the pathophysiology of acute CNS infection by HIV-1, histological studies of specimens from HIV-1–infected humans and SIV-infected rhesus macaques have demonstrated that lymphocytes and monocytes migrate into the brain early in infection (71, 72). A recent study in SIV-infected macaques indicated that perivascular macrophages, which are frequently replenished by peripheral monocytes/macrophages, are the earliest and primary cell type infected in the CNS (73), but the role of lymphocytes in the development of HIV-associated dementia is still unclear. One might speculate that in vivo infections of transgenic rats could provide some insight into this question since productive infection of monocytes/macrophages and T lymphocytes appeared to be naturally uncoupled in these animals (see below). Also, macrophages and microglia are thought to be crucial for the development of HIV-associated dementia because they are the only resident cells that can be productively infected at high levels in the CNS (for a review, see reference 74). Thus, based on the ex vivo data from hCD4/hCCR5 transgenic rats, it is conceivable that certain aspects of HIV-associated CNS pathology can be recapitulated in this small animal model.

Unlike macrophages, primary T lymphocytes from hCD4/hCCR5-transgenic rats were found to harbor a major posttranscriptional block to HIV-1 replication. The early phase of the viral replication cycle was fully intact, since infections by different R5 pseudotypes yielded abundant signals from luciferase reporter viruses. We have previously established that luciferase expression in this cell context is a useful surrogate for HIV-1 Nef protein expression (19). Although the expression level of this reporter gene in rat T cells was only three to sevenfold lower than that found in human lymphocytes, infected transgenic rat lymphocytes failed to secrete significant concentrations of p24 CA. The nonproductive infection of rat lymphocytes was similarly observed previously following infections by HIV-1/(VSV-G), which also demonstrated that there was no significant de novo synthesis of Gag products in rat lymphocytes despite abundant early, Rev-independent gene expression (19). These findings are consistent with an impaired function of Rev in promoting the nuclear export of unspliced viral transcripts in this cell type. This hypothesis is also supported by a recent study on HIV-1 provirus transgenic rats, which revealed the absence of unspliced viral transcripts from the majority of tissues analyzed (20). Future investigation will be needed to define the exact nature of the block in primary rat T cells affecting the transition from the early to the late phase of the viral replication cycle. In the context of an NL4–3 provirus-transgenic mouse model, macrophages, but not lymphocytes, were found to upregulate the expression of p24 CA and infectious virus after polyclonal stimulation (56, 75). In contrast, another study on JR-CSF transgenic mice indicated that comparable, low levels of infectious virus were being released from primary monocytes, as well as T and B lymphocytes (76). It is thus unclear whether mice share this cell type-specific restriction to HIV-1 replication evident in rats. On one hand, the failure of HIV-1 replication to proceed beyond early gene expression in primary rat T lymphocytes may help to dissect mechanisms of $CD4^+$ T cell depletion in an in vivo model. Specifically, transgenic rat T cells should provide a tool to study the individual quantitative and qualitative contributions of hCCR5-mediated signaling to HIV-1 pathology (60, 61, 77, 78) and to study the importance of early HIV-1 gene products for T cell pathology, since infection results in the expression of these products at physiological levels in the absence of structural HIV-1 gene products. For example, the early HIV-1 Nef protein itself has been suggested to harbor a major determinant of pathogenicity for an AIDS-like disease induced by transgenic expression in mice (79). On the other hand, the identification of cellular factors that are essential for HIV-1 replication and that may surmount restrictions in primary rat T lymphocytes would both provide a better understanding of the molecular aspects of HIV-1 replication and increase the range of applications of a transgenic rat model.

In a first set of studies seeking to establish whether or not rat cells in vivo are permissive for HIV-1 infection, systemic challenge with R5 viruses yielded definitive evidence for viral infection in cells from spleen, peripheral blood, and thymus. Cells from hCD4/hCCR5 transgenic, but not nontransgenic rats, contained episomal 2-LTR circles as well as integrated proviral DNA. Early viral gene expression, represented by an EGFP reporter expressed from the nef gene locus, was found in $rCD4^+$ lymphocytes and macrophages in double-transgenic rats. Interestingly, the frequency of infected $CD4^+$ lymphocytes in the spleen of transgenic rats (0.14–0.18%) was in the same range as those described in spleen sections from AIDS patients (0.09–0.64%) in a recent report (80). Our results constitute the

first demonstration of a quantitative HIV-1 infection of lymphoid tissues in a transgenic rodent.

It has been suggested that 2-LTR circles are labile, both in vitro and in vivo, relative to integrated viral genomes, and thus indicative of a recent cellular infection event (45). Further investigations will be required to define the nature of the persistence of 2-LTR circles in transgenic rats, and specifically whether or not this persistence reflects multiple rounds of replication. The detectable, albeit low, levels of 2-LTR circles in spleen and thymus samples from transgenic rats 6 mo after inoculation are encouraging in this respect. In addition, we found that transgenic rats challenged with YU-2 intraperitoneally had detectable plasma viremia up to 7 wk after infection. Although the sample size is small and the levels of viremia were modest, these findings imply that a productive infection may occur in this rodent model. Also, detectable plasma viremia in one transgenic rat (#521) correlated in time with an observed neurological event before an unexplained, spontaneous death.

Taken together, our data demonstrate that hCD4/hCCR5 transgenic rats are promising candidates for a small animal model of HIV-1 infection. Whether the productive infection of cells from the monocyte/macrophage lineage will be sufficient for sustained viral loads in vivo and/or induction of HIV-induced pathogenesis in the present form of the model is currently under investigation. A recent study reported that HIV-1 provirus transgenic rats developed HIV-related pathologies and immunologic dysfunction (20). This indicates that HIV-1 gene expression in this species may be sufficient to recapitulate certain aspects of the disease in humans, and provides an additional basis for optimism that HIV-related pathology will be found in the hCD4/hCCR5-transgenic rat model. Such a system for de novo infection would provide unique experimental opportunities for elucidating aspects of viral transmission, viral dynamics, HIV-1 pathogenesis, immune responses, and efficacy of therapeutic strategies aimed at diverse aspects of the viral life cycle. A systematic assessment of in vivo infections in hCD4/hCCR5-transgenic rats will be needed to define in more detail the features and limitations of this model system, as well as opportunities for enhancement based on further genetic and biological manipulation.

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EXHIBIT B

An HIV-1 transgenic rat that develops HIV-related pathology and immunologic dysfunction

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Contributed by R. C. Gallo, June 11, 2001

We report, to our knowledge, the first HIV type 1 (HIV-1) transgenic (Tg) rat. Expression of the transgene, consisting of an HIV-1 provirus with a functional deletion of *gag* and *pol*, is regulated by the viral long terminal repeat. Spliced and unspliced viral transcripts were expressed in lymph nodes, thymus, liver, kidney, and spleen, suggesting that Tat and Rev are functional. Viral proteins were identified in spleen tissue sections by immunohistochemistry and gp120 was present in splenic macrophages, T and B cells, and in serum. Clinical signs included wasting, mild to severe skin lesions, opaque cataracts, neurological signs, and respiratory difficulty. Histopathology included a selective loss of splenocytes within the periarterial lymphoid sheath, increased apoptosis of endothelial cells and splenocytes, follicular hyperplasia of the spleen, lymphocyte depletion of mesenteric lymph nodes, interstitial pneumonia, psoriatic skin lesions, and neurological, cardiac, and renal pathologies. Immunologically, delayed-type hypersensitivity response to keyhole limpet hemocyanin was diminished. By contrast, Ab titers and proliferative response to recall antigen (keyhole limpet hemocyanin) were normal. The HIV-1 Tg rat thus has many similarities to humans infected with HIV-1 in expression of viral genes, immune-response alterations, and pathologies resulting from infection. The HIV-1 Tg rat may provide a valuable model for some of the pathogenic manifestations of chronic HIV-1 diseases and could be useful in testing therapeutic regimens targeted to stages of viral replication subsequent to proviral integration.

HIV type 1 (HIV-1) infection is marked by a constellation of pathologies in addition to a variety of secondary infections (1). These include central and peripheral neuropathies (2), follicular lymph node hyperplasia (particularly early in infection; ref. 3), lymphoid depletion (at least in part involving apoptosis; refs. 4 and 5), interstitial pneumonitis (especially in pediatric cases; refs. 6 and 7), tubulointerstitial nephritis, glomerulosclerosis (8–10), wasting (11), and heart disease (12).

To establish a small-animal model for HIV-associated pathologies, several HIV transgenic (Tg) mice have been established. A *Tat* transgene was reported to cause dermal lesions resembling Kaposi sarcoma (13) or lymphoid hyperplasia in spleen and lymph nodes, and B cell lymphoma (14) when expression was regulated by the viral long terminal repeat (LTR) or a heterologous promoter, respectively. Expression of a *nef* transgene driven by the HIV-1 LTR also resulted in dermal lesions (15). More recently, Tg mice expressing *nef* under the control of a human T cell-specific promoter and mouse enhancer developed immunodeficiency with loss of T cells and alterations of T cell function (16). Tg mice also have been constructed with a *gag-pol*-deleted HIV-1 provirus regulated by the viral promoter and are characterized by wasting/runting, psoriatic skin lesions, cataracts, and nephropathy. Some HIV-1 gene expression occurs in most tissues, but is highest in skin and muscle (17, 18).

Despite replicating some of the pathologies in humans, viral expression in many existent HIV-1 Tg mouse models is regulated either by heterologous promoters, resulting in expression in atypical tissues, or by the viral promoter, which gives the highest expression in skin. The viral promoter does not function normally in mice, in part because mouse cyclin T does not interact functionally with Tat (19). We report here the construction of Tg rats that contain a *gag-pol*-deleted HIV-1 provirus regulated by the viral promoter. Unlike mice with the same transgene, efficient viral gene expression occurs in lymph nodes, spleen, thymus, and blood, suggesting a functional Tat. The Tg rat thus may offer significant advantages as a noninfectious small-animal model of HIV-1 pathogenesis.

Materials and Methods

Construction of HIV-1 *gag-pol*-Tg Rat. The construction of the plasmid from which the transgene was derived has been described (17). Briefly, a 3-kbp *Sph*I-*Msc*I fragment encompassing the 3′ region of *gag* and the 5′ region of *pol* was removed from pNL4–3, an infectious proviral plasmid, to make the noninfectious HIV-$1^{gag\text{-}pol}$ clone pEVd1443. A 7.4-kbp *Eae*I-*Nae*I fragment containing the provirus and host cell flanking regions was microinjected into fertilized one-cell Sprague–Dawley × Fisher 344/NHsd F1 eggs as described (20). The experimental protocol was approved by the University of Maryland Biotechnology Institute Institutional Animal Care and Use Committee.

Macrophages and B and T Cell Isolation. Splenocytes were purified on Histopaque-1083 (Sigma), counted, divided into 3 aliquots of 1.5×10^7 cells, and stained with primary mAbs by standard procedure (21). Each aliquot was labeled for 45 min at room temperature with a 1:100 dilution of anti-rat mAbs ED1 (MCA341R; Serotec), CD45RA (MCA340R; Serotec), or CD3 (22011D; PharMingen), which are specific for rat macrophages and B and T cells, respectively. Cells (10^7) were magnetically labeled with 20 μl of magnetic activated cell sorting (MACS) rat anti-mouse IgG1 (471-02) or goat anti-mouse IgG (484-02) (Miltenyi Biotec, Auburn, CA) for 15 min at 6–12°C. The magnetically labeled cell suspensions were separated by positive selection on MS+ separation columns placed in a VarioMACS magnet (Miltenyi Biotec), using the manufacturer's recommendations.

Abbreviations: DTH, delayed type hypersensitivity; H&E, hematoxylin and eosin; HIV-1, HIV type 1; KLH, keyhole limpet hemocyanin; PALS, periarterial lymphatic sheath; Tg, transgenic; LTR, long terminal repeat; Th, T helper.

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Histology, Immunochemistry, and Apoptosis. Tissues from Tg and non-Tg littermates and Fisher 344/NHsd Sprague–Dawley control rats were fixed in 10% neutral buffered formalin and embedded in paraffin. Five-microgram tissue sections were used for hematoxylin and eosin (H&E) staining, immunohistochemistry, and apoptosis assays. A modified avidin/biotin method was used for immunohistochemical localization of HIV gene products (22). Paraffin sections were processed as described, exposed to antigen unmasking solution (Vector Laboratories), incubated in 3% H_2O_2 for 20 min, and treated with avidin/biotin blocking solution (Vector Laboratories) and nonimmune sera appropriate for blocking the secondary Ab at a 1:5 dilution. Primary Abs (all from Advanced Biotechnologies, Columbia, MD, except where noted) included HIV-1 gp120 goat antiserum (13-202-000), diluted 1:50 and 1:100; HIV-1 rabbit anti-HIV gp120 antiserum (13-204-000), diluted 1:100; mouse anti-HIV-1 gp120 mAb (NEN, Boston, MA; NEA 9301), diluted 1:150; mouse anti-HIV-1 Tat mAb (13-162-100), diluted 1:50 and 1:100; and mouse anti-HIV-1 Nef (13-152-1000), diluted 1:50 and 1:100. Biotinylated secondary Abs were incubated for 2 h at room temperature with anti-mouse IgG (rat-absorbed), anti-rabbit IgG, and anti-goat IgG (Vector Laboratories), at dilutions of 1:200–500, and labeled with Vecta Stain Elite ABC kit (Vector Laboratories), followed by addition of 3,3′-diaminobenzidine tetrahydrochloride (DAB) peroxidase (Sigma) or the 3-amino-9-ethylcarbazole (AEC) substrate system (Dako) to visualize the immunolabel.

Immunization of Rats. Tg and Fisher 344/NHsd control rats were immunized i.p. with 100 μg of keyhole limpet hemocyanin (KLH; Pierce) with complete Freund's adjuvant (Difco). Four weeks later, sera were collected, and anti-KLH end-point titers were determined by ELISA. Microtiter plates (NUNC MaxiSorp, Roskilde, Denmark) were coated with 2 μg/ml KLH overnight at 4°C. Plates were washed, blocked with 5% milk powder, and incubated with serially diluted rat sera at 37°C for 2 h. Bound IgG was detected with horseradish peroxidase-conjugated anti-rat IgG Ab. For detection of delayed type hypersensitivity (DTH), rats were challenged intracutaneously 4 weeks after immunization with 50 μg of KLH, and the size of the induration was measured 24 h later.

Proliferation Assays. Splenocytes (2.0×10^5) were cultured in 0.2 ml of RPMI medium 1640 with 10% (vol/vol) FBS in a microtiter plate with 50 μg/ml of KLH or 10 μg/ml of phytohemagglutinin. The cells were pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; NEN) for 18–24 h and harvested onto glass fiber filters with a Micron 96-cell harvester (Skatron, Lier, Norway). Incorporation of [^{3}H]thymidine was measured with a liquid scintillation counter. All experiments were in triplicate.

Statistics. The mean number of apoptotic splenocytes per high power field, DTH measurements, and KLH end-point titers between phytohemagglutinin-stimulated and unstimulated cells from Tg and normal offspring were compared by using an independent Student's t test for samples exhibiting normally distributed values. A Wilcoxon rank sum test was used for samples exhibiting non-normal distribution. Two-tailed P values were considered significant at $P < 0.05$.

Results

Preliminary Analysis of HIV-1 Tg Rats. One female founder was produced that had very opaque cataracts. Mating with a wild-type Fischer 344/NHsd produced several F1 Tg and normal offspring. F1 offspring had very opaque (line 1) or very mild cataracts (line 2). The Tg animals were grouped according to cataract phenotypes, and DNA was prepared from tail tips, digested with *Eco*RI, and analyzed by Southern blotting (ref. 23;

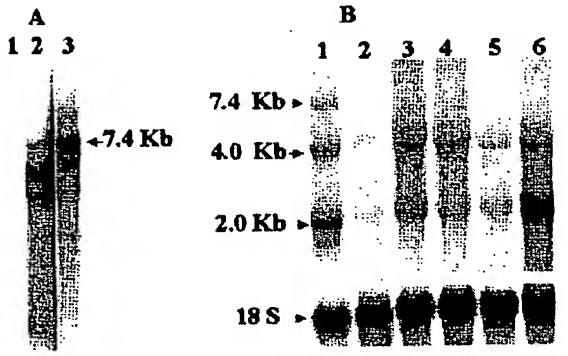

Fig. 1. Detection and expression of transgene. (*A*) Detection of transgene. A Southern blot was performed on *Eco*RI-digested DNA from tail snips of a normal rat (15 μg; lane 1) or Tg rats with mild (20 μg; lane 2) or very opaque (5 μg; lane 3) cataracts. The blot was hybridized to an α-^{32}P-labeled 7.4-kb *Eae*I-*Nae*I fragment from pEVd1443. (*B*) Expression of transgene RNA. A Northern blot was performed on total RNA isolate from various tissues and analyzed by Northern blot as described (32). HIV-specific transcripts (7-, 4-, and 2-kb) were detected with an α-^{32}P-labeled 1.3-kbp (*Bgl*II/*Bgl*II) fragment from pHXB2, containing gp41 and *nef* coding regions. HIV transcripts were quantified with a Storm 840 PhosphorImager (Molecular Dynamics) and normalized for hybridization to 18S rRNA by using an appropriate probe. RNA was from the axillary (lane 1) and mesenteric (lane 2) lymph nodes, thymus (lane 3), liver (lane 4), kidney (lane 5), and spleen (lane 6) of transgenic rat with highly opaque cataracts. The positions of the 7.0-kb full-length mRNA, 4-kb singly spliced *env* mRNA, and 2-kb multiply spliced mRNA are indicated.

Fig. 1*A*). *Eco*RI cuts the transgene once. As shown in lanes 2 and 3 of Fig. 1*A*, the hybridization patterns of DNA representing the two phenotypes differed. Both gave bands corresponding to a full-length transgene, as indicated by the arrow, suggesting that both integration sites contained two or more tandem copies of the transgene. Other bands, presumably representing transgene–host junction bands or head-to-tail multiple tandem integrated transgenes, differed, suggesting that the transgene had integrated at two independently segregating sites, one resulting in the opaque cataract phenotype and the other in mild cataracts. A brother–sister mating of F1 Tg rats from line 2 produced offspring with mild cataracts only. Southern blots of *Eco*RI-digested DNA from offspring were identical to those of the parents. As judged by the relative intensity of hybridization to Southern blots of Tg DNA compared with serial dilutions of known amounts of plasmid DNA quantified with a PhosphorImager (Molecular Dynamics), Tg rats from line 1 contained 20–25 copies, whereas those of line 2 contained only a few. Subsequent studies focused only on line 1 HIV-1 Tg rats.

HIV-1 Transgene Expression. RNA from numerous tissues was analyzed for viral transcripts. Expression levels were generally highest in lymph nodes, spleen, kidney, and thymus. A representative Northern blot of lymph node RNA (Fig. 1*B*) shows three viral-specific bands, representing full-length 7.4-kb mRNA, 4.0-kb singly spliced Env mRNA, and multiply spliced 2-kb mRNA transcripts for Nef, Tat, and Rev. The presence of Tat mRNA in the 2-kb band from spleen RNA was confirmed by reverse transcription–PCR amplification and DNA sequence analysis (not shown). Formalin-fixed paraffin-embedded 5-μm sections of spleen from line 1 (Fig. 1*B*) Tg rats were analyzed by immunohistochemistry for Env gp120, Nef, and Tat. All three proteins were evident in cells within the red and white pulp of the spleen (Fig. 2*A–C*). Gp120 was present in cellular lysates of spleen-derived macrophages and B and T cells as judged by

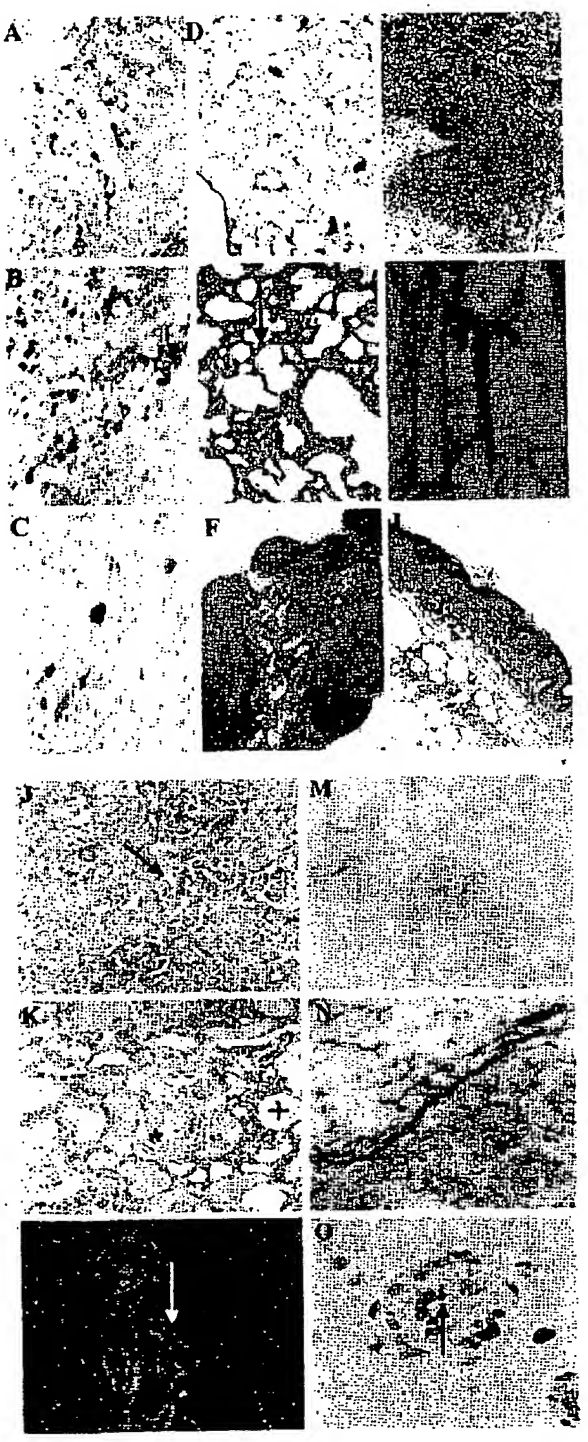

Fig. 2. Pathology and transgene expression. A section of Tg rat spleen was stained for HIV gp120 (*A*), Nef (*B*), and Tat (*C*). Cytoplasmic staining (dark brown) is evident for all three proteins (original magnification = ×60). H&E-stained sections of control (*D*) and Tg (*E*) lung are shown (×20). Arrow in *E* indicates area of interstitial thickening. H&E-stained sections of control (*F*) and Tg (*G*) mesenteric lymph node (×10) are shown. Note normal follicle (*, *F*) and hemorrhage, lymphoid depletion, and vascular proliferation in Tg section (*G*). Gross tail and foot lesions (*H*) and H&E-stained Tg skin (×60). Note psoriatic skin lesions with hyperkeratosis and mononuclear cell infiltrate (arrow, *I*). H&E-stained control kidney shows normal glomerulus (*) and renal tubule (arrow, *J*); Tg kidney shows focal glomerulosclerosis (*) and tubulointerstitial disease (+, *K*; ×60). H&E-stained heart section (*L*) shows myocardial inflammation with mononuclear cell infiltration (arrow) (×60). Astrocytes from normal (*M*) or Tg (*N*) brains were stained for glial fibrillary acidic protein (GFAP; ×40). Staining for GFAP was as reported (22), using anti-GFAP Ab (U703B; Dako). Staining of normal brain was limited, but Tg brain was heavily stained, indicating reactive gliosis, a marker for central nervous system damage. (*O*) Blood vessels in Tg brain were stained with ApopTag (×60); arrow indicates vascular endothelial apoptosis.

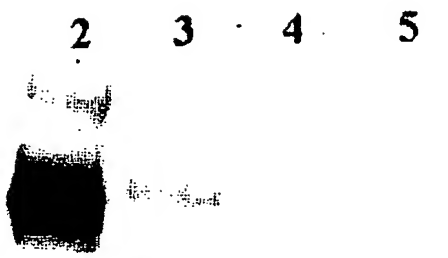

Fig. 3. Transgene expression in splenocytes. Seven micrograms of protein from extracts of spleen-derived macrophages (lane 3), B cells (lane 4), or T cells (lane 5) was fractionated on a 4–12% NuPage gel (NOVEX, San Diego) and analyzed by Western blot, using a 1:100 mouse anti-HIV-1 gp120 mAb (NEA 9301, NEN). Proteins were visualized by using the enhanced chemiluminescence (ECL) plus Western blotting system (Amersham Pharmacia). Lane 1 contained extract from a normal control spleen, and lane 2 contained 30 ng of recombinant gp120 as a positive control.

Western blotting (Fig. 3). The animals were also antigenemic. As measured by ELISA, sera contained 100–200 pg/ml of gp120 (Table 1). Thus, in marked contrast to Tg mice with the same proviral transgene (17, 18), Tg rats express viral gp120 in macrophages and B and T cells and shed it into peripheral blood.

Pathology of Tg Rats. Cataract formation ranged from mild to highly opaque. Some Tg animals (>50%) presented with highly angiogenic corneas. Their lenses had marked vacuolization, liquefaction, and fragmentation (not shown). In addition to cataracts, HIV-1 Tg rats developed many clinical manifestations of AIDS by 5–9 months of age, including weight loss, neurological abnormalities, and respiratory difficulty. Generally, neurological abnormalities were characterized by circling behavior and hind-limb paralysis. Often (>70%), the lung showed mild interstitial pneumonia characterized by mild to moderate interstitial fibrosis and mononuclear cell infiltration (Fig. 2 *D* and *E*). Mesenteric lymph nodes (>40%) were generally enlarged, and many histological sections showed lymphoid depletion and fibrosis (Fig. 2 *F* and *G*). Many Tg rats (>20%) had focal to extensive ulcerative skin lesions (Fig. 2 *H* and *I*). Histologically, the lesions were hyperkerototic, with elongation of the rete ridges. The kidneys from clinically ill rats (>90%) were diffusely pale, and the capsular surface was pitted, similar to what is seen with HIV-1 associated-nephropathy. H&E-stained kidney sections showed a spectrum of renal disease varying from mild to severe. Generally, glomeruli in Tg rats contained increased periodic acid/Schiff reagent (PAS)-positive material, with either segmental or global sclerosis. Some glomeruli showed mesangial hypercellularity and enlargement of visceral epithelial cells. Silver staining confirmed that the PAS-positive tissue within the

Table 1. Serum gp120 in Tg rats

Tg rats	gp120, pg/ml*
Tg no. 1	151
Tg no. 2	129
Tg no. 3	121
Tg no. 4	85
Tg no. 5	172
Tg no. 7	235
Tg no. 8	164
Tg no. 9	217

The expression of gp120 envelope protein in the serum was assayed by ELISA antigen capture assay. The average gp120 concentration in the serum of hemizygous rats (n = 9) was 141 pg/ml (A).

*Capture ELISAs were done in triplicate on Tg rats with negative controls (n = 8). A mean adjusted value (76.6) for negative controls was subtracted.

glomeruli was composed of matrix material (not shown). The renal tubules showed microcystic tubular and tubulointerstitial pathology characterized by tubular degeneration, interstitial fibrosis, and mononuclear cell infiltration (Fig. 2 *J* and *K*). The heart generally was round and pale in color (not shown). Some hearts from Tg rats showed evidence of endocarditis and myocardial inflammation characterized by necrosis, mononuclear cell infiltrates, and multiple vascular abnormalities (Fig. 2*L*).

Tg rat brains with or without clinical neurological signs were grossly unremarkable. However, pathologic changes were evident in rats with clinical signs. Capillaries and endothelial cells presented with atypical changes, such as microscopic hemorrhages and endothelial cell apoptosis, in a multifocal distribution (Fig. 2*O*). Foci of gliosis together with neuronal cell death were noted, particularly in animals with clinically observable signs (Fig. 2*N*). For comparison, Fig. 2*M* shows a normal section of brain. Although the distribution of these changes seemed random, when they occurred with increased severity in focal areas of the brain, corresponding neurological deficits were noted. For example, animals with motor problems presented with greater severity of changes in the caudate putamen and substantia nigra.

Tg rat spleen was generally normal in size. Spleen tissue sections showed a loss of splenocytes within the periarterial lymphatic sheath (PALS), expansion of the marginal zones, follicular hyperplasia, and apoptosis of endothelial cells and splenocytes (Fig. 4 *A* and *B*). Apoptosis of splenocytes was evaluated by counting ApopTag (Intergen, Purchase NY)-positive cells per high-powered field in 5-month-old male rats and was significantly elevated compared with age- and sex-matched non-Tg controls ($P < 0.01$; Fig. 4*C*).

Immune Function of HIV-1 Tg Rats. To evaluate immune function, Tg rats and age-matched controls were immunized with KLH, and the resultant KLH-specific DTH responses and anti-KLH Ab titers were determined. The DTH response was determined from the extent of induration present at 24 h. The induration was significantly lower relative to controls ($P < 0.01$; Table 2). In contrast, there was no significant difference in anti-KLH-specific Ab titers, suggesting that the Tg rats have an abnormal T helper (Th)-1 response but normal Th2 function. The mean proliferative response of spleen cells from adult Tg rats to nonspecific mitogen (phytohemagglutinin) was significantly increased after 6 days compared with littermate controls ($P = 0.0062$; Table 3). However, there were no statistical differences in the T cell proliferative response of splenocytes from vaccinated rats to a recall antigen (KLH).

Discussion

We report, to our knowledge, the first HIV-1 Tg rat. The transgene, consisting of an HIV-1 provirus deleted for *gag* and

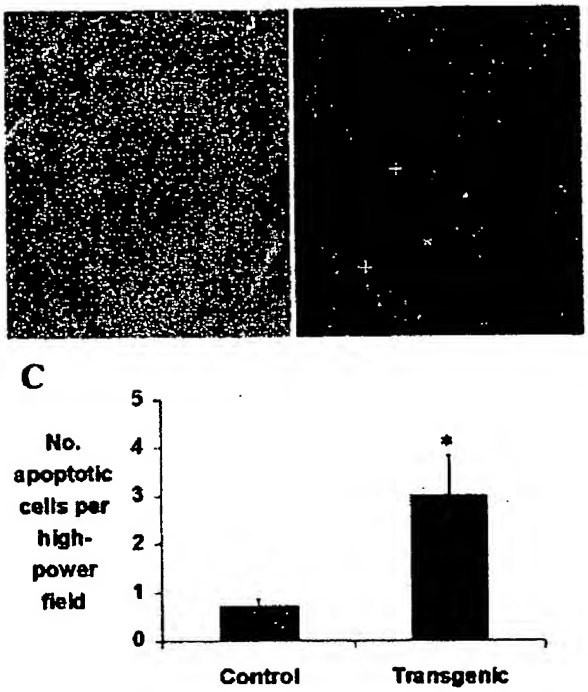

Fig. 4. Pathology and apoptosis in Tg rat spleen. (*A*) H&E-stained control spleen. Note T cell region (PALS) (*) and B cell region (marginal zone) (→) (original magnification ×60). (*B*) H&E-stained Tg rat spleen (×60). Note loss of T cells within the PALS (*). The two + show the marginal zone. (*C*) Five-microgram tissue sections were assayed *in situ* for apoptosis by using an ApopTag kit. Spleen sections from 5-month-old male Tg and Fisher 344/NHsd control rats were taken from 3 animals per group, and apoptotic cells from each section were enumerated 3 times at ×400 by using a Nikon Labophot-2 light microscope. The entire tissue section was counted by using a stage micrometer to pick successive nonoverlapping fields. The * shows the statistically significant difference between HIV-1 transgenic rats and age-matched controls.

pol, is expressed in lymphoid tissues, including lymph nodes, spleen, thymus, and blood. The Tg rats exhibit pathologies and immune irregularities characteristic of HIV-1 infection of humans.

The tissue-specific pattern of viral gene expression is of interest. To model viral gene expression in infected humans, we

Table 2. Antibody and DTH responses to KLH in HIV-1 Tg rats

Exp. group	Observations	Anti-KLH Ab titer	Anti-KLH DTH, mm diameter	SE
Tg*	12	7.4	NA	0.19
Control*	6	7.9	NA	0
Tg†	10	NA	3.5‡	0.7
Control†	6	NA	10.8	1.4

NA, not applicable.

*Tg and non-Tg rats were immunized i.p. with 100 μg of KLH with complete Freund's adjuvant. Four weeks later, sera was collected, and end-point anti-KLH Ab titers were determined by ELISA. The results are expressed as mean ± SE (logarithm 10) of end-point titers.

†Immunized rats were challenged with 50 μg of KLH intracutaneously, and the size of the induration was measured 24 h later. Results are expressed as mean ± SE of diameter of induration (mm).

‡Indicates a statistical significance between HIV-1 Tg and non-Tg rats.

Table 3. Proliferative response of splenocytes from Tg

Exp. group	Observations	[³H]thymidine incorporation of stimulated spleen cells, Δcpm		
		PHA	KLH	SE
Tg*	8	28,289‡	NA	7,201
Control*	5	2,138	NA	1,070
Tg†	4	NA	89,202	18,147
Control†	3	NA	92,475	24,260

NA, not applicable.

*Spleen cells (2×10^5) from HIV-1 Tg or non-Tg rats were cultured with 10 μg/ml of PHA at 37°C for 6 days.

†Spleen cells (2×10^5) from HIV-1 Tg or non-Tg rats immunized with KLH 4 weeks previously were cultured with 50 μg/ml KLH at 37°C for 5 days. All cells were labeled with 1 μCi [³H]thymidine for the last 18–24 h, harvested, and the incorporated radioactivity counted. The results are expressed as mean ± SE of thymidine incorporation in triplicate cultures.

‡Indicates a statistical significance between Tg and non-Tg rats.

used the viral LTR to regulate the transgene. LTR-regulated gene expression depends on transactivation by the viral Tat protein, which requires cellular cyclin T as a cofactor. Mouse cyclin T is not functional with Tat, and LTR-driven expression in Tg mice differs from expression in humans, with the highest levels in skin and muscle. In contrast, viral genes are efficiently expressed in lymphoid tissues of Tg rats, similar to infected humans, suggesting that rat cyclin T is functional. A similar suggestion has been made by Bieniasz and Cullen (24). Three sizes of viral transcripts can be detected, including a 7.4-kb unspliced mRNA, a 4.3-kb singly spliced (*env*) mRNA, and a 2-kb multiply spliced (*tat*, *rev*, and *nef*) mRNA, similar to infected human cells, suggesting that Rev, a viral protein that regulates splicing, can also function in rats.

Clinical manifestations, similar to those in humans infected with HIV, are evident in the Tg rats, including neurological changes, respiratory difficulty, and cataracts. The neuropathology includes reactive gliosis, neuronal cell loss, lymphocyte infiltration, and alteration of endothelial cells with loss of blood–brain barrier integrity. There is a degeneration of peripheral nerves and skeletal muscle atrophy similar to that in infected humans. The Tg rat could be a useful model of HIV–central nervous system interactions, because the neuroanatomy, neurophysiology, neuropathology, and behavior of rats are well studied.

Pneumonitis associated with HIV-1 infection is common in pediatric cases of AIDS (6); however, its pathogenesis is not well understood. Most cases of pneumonia in people infected with HIV-1 are characterized by lymphocytic interstitial pneumonitis. Respiratory difficulty in the Tg rats is correlated with a mild expansion of the lung interstitium by a mononuclear infiltrate. The pneumonia in the Tg rats is unlikely to be caused by opportunistic infections, because of their pathogen-free status, sterile housing conditions, and certified diet, suggesting that pathogenesis is mediated directly by the HIV-1 transgene.

Cardiac disorders including myocarditis and cardiomyopathies have been reported in patients infected with HIV, but their etiopathogenesis is uncertain (12, 25, 26). The cardiac pathology seen in the Tg rats is grossly and microscopically similar to that in infected people, suggesting that this could be a useful model for the study of HIV-associated cardiac disease. There is controversy about the role of HIV as the primary etiology of cardiac pathology; opportunistic infections, cardiotoxic substances, nutritional deficiencies, and autoimmune reactions have been suggested as contributing factors. Because the Tg rats are pathogen-free, the observed cardiac abnormalities are likely caused by viral gene product activity rather than opportunistic infections.

Renal abnormalities are frequently seen in AIDS, especially in African-American patients (8, 27, 28). The most typical is HIV-1 associated-nephropathy (HIVAN), occurring in 10–15% of all infected African-American patients. HIVAN includes proteinuria, nephrotic syndrome, focal or segmental glomerulosclerosis, and tubulo-interstitial disease, rapidly progressing to end-stage renal disease. The pathogenesis is not completely understood but likely involves the direct effects of HIV-1 and cytokines such as basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-β (28) that are secreted by infected cells (29). The Tg rat offers a potential model for HIVAN and related conditions.

HIV-1 Tg rats have selective immune abnormalities. There is a markedly decreased DTH response but Ab titers and the proliferative response of splenocytes to KLH antigen is normal. Lymphoid tissues manifest an AIDS-like histopathology, including lymphocyte depletion in lymph nodes, severe splenic hyperplasia with loss of cells around the PALS, and increased apoptosis of splenocytes. Viral proteins are expressed in spleen T and B cells and monocytes. This hyperplasia and cell loss may be caused in part by abnormal trafficking of cells or by abnormal or selective proliferation and loss of lymphocyte subsets. It has recently been shown in mice that $CD8\alpha^+$ dendritic cells and naïve and Th1 T cells concentrate within the PALS, whereas $CD8\alpha^-$ dendritic cells and Th2 cells form loose rings around the outer PALS in close proximity to the B cells (30, 31). It is tempting to speculate that the loss of T cells around the PALS and in lymph nodes, the increased apoptosis in spleen, and the diminished DTH reaction are the results of a common mechanism affecting Th1 development or trafficking. Taken together with the pattern of pathologies that occur, the HIV-1 Tg rat is potentially a highly useful small-animal model for AIDS pathogenesis.

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EXHIBIT C

HIV-susceptible transgenic rats allow rapid preclinical testing of antiviral compounds targeting virus entry or reverse transcription

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The current testing of anti-HIV drugs is hampered by the lack of a small animal that is readily available and easy to handle; can be infected systemically with HIV type 1 (HIV-1); harbors the major HIV-1 target cells in a physiological frequency, organ distribution, and activation state; and is established as a pharmacological model. Here, we explored the potential of outbred Sprague–Dawley rats that transgenically express the HIV-1 receptor complex on CD4 T cells and macrophages as a model for the preclinical evaluation of inhibitors targeting virus entry or reverse transcription. The concentrations of the peptidic fusion inhibitor enfuvirtide or the nonnucleoside reverse transcriptase inhibitor efavirenz required to inhibit HIV-1 infection of cultured primary CD4 T cells and macrophages from human CD4 and CCR5-transgenic rats differed by no more than 3-fold from those required for human reference cultures. Prophylactic treatment of double-transgenic rats with a weight-adapted pediatric dosing regimen for either enfuvirtide (s.c., twice-daily) or efavirenz (oral, once-daily) achieved a 92.5% or 98.8% reduction, respectively, of the HIV-1 cDNA load in the spleen 4 days after i.v. HIV-1 challenge. Notably, a once-daily dosing regimen for enfuvirtide resulted in a ≈5-fold weaker inhibition of infection, unmasking the unfavorable pharmacokinetic characteristics of the synthetic peptide in the context of an efficacy trial. This work provides proof of principle that HIV-susceptible transgenic rats can allow a rapid and predictive preclinical evaluation of the inhibitory potency and of the pharmacokinetic properties of antiviral compounds targeting early steps in the HIV replication cycle.

animal model | drugs | efficacy trial

Despite major achievements in HIV pharmacotherapy over the past decade, there remains an urgent need for more potent, less toxic, and conceptually novel antiretroviral drugs. The process of anti-HIV drug development is facilitated by efficient and predictive models capable of selecting the best compounds at each decision point. The preclinical validation in animal models is a critical complementation of *in vitro* efficacy and toxicity assays to facilitate the prioritization of antiviral compounds for phase I clinical trials. This notion is emphasized by the fact that a number of compounds with relatively good efficacy against HIV-1 *in vitro*, including dextran sulfate and the nonnucleoside reverse transcriptase inhibitor (NNRTI) atevirdine, have failed in clinical trials (1, 2).

Native mice and rats are nonpermissive for HIV-1 (3). The preclinical testing of anti-HIV drugs has thus concentrated on various xenotransplant models, in which human hematopoietic cells or tissues are transplanted into immunodeficient strains of mice. These models include severe combined immunodeficiency (SCID) mice, in which human fetal thymus and liver are simultaneously implanted under the kidney capsule [SCID-hu (Thy/Liv) model] (3, 4). Three to five months posttransplantation, successfully vascularized grafts can be directly challenged with HIV-1 during a second surgical procedure. Three to six weeks postinfection, end point analyses of grafts include the quantification of the proviral load, CD4 T cell depletion, and overall graft viability, permitting an assessment of drug efficacy and toxicity (5). As alternative yet related mouse models for HIV-1 infection, human peripheral blood lymphocytes (PBL) can be adoptively transferred i.p. into either SCID mice (hu-PBL-SCID model) or into sublethally irradiated mice reconstituted with SCID bone marrow (Trimera mouse model) (4, 6). However, all of these xenotransplant models do not recapitulate the full spectrum, frequency, and physiological organ distribution of HIV-1 target cells found in humans and are technically very challenging and time-consuming. The simian immunodeficiency virus (SIV)/HIV (SHIV) rhesus macaque model has been of some value for preclinical testing (7–11), but non-human primate studies are severely limited by ethical concerns and high cost, resulting in small animal group sizes and restricted accessibility.

In a conceptually different approach to the development of an HIV animal model (12), we have recently generated immunocompetent transgenic rats on an outbred Sprague–Dawley background that express the HIV-1 receptor complex selectively on CD4 T cells, macrophages, and microglia (13). In humans, these cell types are the primary targets for productive HIV infection. *Ex vivo* cell cultures from human CD4 and CCR5 (hCD4/hCCR5)-transgenic rats were susceptible to infection by HIV-1, leading to expression of early viral gene products. Furthermore, primary macrophages and microglia supported a productive infection by various recombinant and primary strains of HIV-1, including YU-2, JR-CSF, Ada-M, Ba-L, as well as the primary, patient-derived HIV-1 isolates C1 and O3. In contrast, T cell cultures from double-transgenic rats did not allow a spreading infection (13). After systemic HIV-1 challenge, lymphatic organs from hCD4/hCCR5-transgenic rats contained different HIV-1 cDNA species and early viral gene products, demonstrating HIV-1 susceptibility *in vivo*. Specifically, CD4 T lymphocytes and macrophages residing in spleen, thymus, as well as in peripheral blood from hCD4/hCCR5-transgenic rats expressed EGFP from the *nef* locus after infection with a replication-competent HIV-1 reporter virus. Furthermore, double-transgenic rats infected with the CCR5-using strain HIV-1_{YU-2} displayed a low-level plasma viremia up to 7 weeks postchallenge as well as two jointed LTR (2-LTR) circles in spleen and thymus 6 months postinfection, consistent with several rounds of low-level replication (13). These rats have been valuable for exper-

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The authors declare no conflict of interest.

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Abbreviations: BlaM-Vpr, β-lactamase-Vpr chimeric fusion protein; hCD4/hCCR5, human CD4 and CCR5; HIV-1, HIV type 1; 2-LTR, two joined LTRs; NNRTI, nonnucleoside reverse transcriptase inhibitor; SCID, severe combined immunodeficiency; VSV-G, vesicular stomatitis virus type G.

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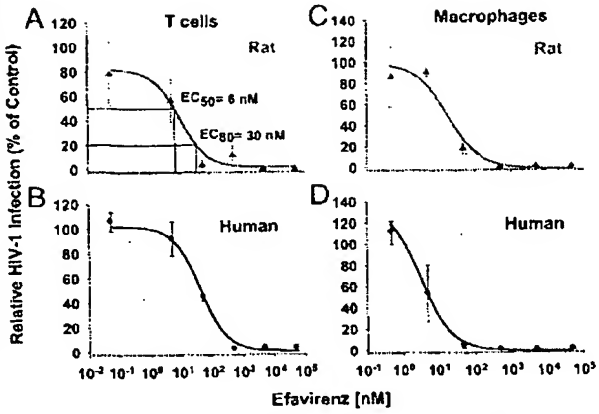

Fig. 1. Dose–response curves for the antiviral potency of the NNRTI efavirenz in *ex vivo* cultures of primary T cells and macrophages from hCD4/hCCR5-transgenic rats and humans. (*A* and *B*) Activated T cells from double-transgenic rats and human donors were pretreated with efavirenz for 1 h and subsequently challenged with VSV-G HIV-1_{NL4-3} EGFP viruses. (*C* and *D*) Macrophages from double-transgenic rats and human monocyte-derived macrophages were pretreated with efavirenz overnight and then challenged with VSV-G HIV-1_{NL4-3} EGFP viruses. On day 3 postinfection, the percentage of EGFP-positive cells was scored by flow cytometry. The percentage of infected cells in untreated controls was set at 100%, and relative levels of HIV-1 infection are shown. Each experiment was performed in triplicate, and one to four experiments were conducted. Given are arithmetic means ± SD of one experiment. EC_{50} and EC_{80} values were determined by using Prism software (GraphPad, San Diego, CA) and are shown in *A* and in Table 1.

imental studies on aspects of HIV-1- and protease inhibitor-induced peripheral neuropathy (14, 15).

In this work, we explored the suitability of hCD4/hCCR5-transgenic rats to serve as a model for a rapid, quantitative, and predictive evaluation of anti-HIV compounds. *Ex vivo* and *in vivo* proof-of-principle efficacy studies were conducted for two clinically approved antiretroviral drugs, the NNRTI efavirenz (Sustiva) and the peptidic fusion inhibitor enfuvirtide (T-20, Fuzeon).

Results

Anti-HIV Efficacies of an NNRTI or of a Fusion Inhibitor in Primary T Cells and Macrophages from hCD4/hCCR5-Transgenic Rats and Humans Are Comparable. We first investigated potential species differences for anti-HIV drug susceptibility studies by comparing the efficacy of the prototypic NNRTI efavirenz and of the fusion inhibitor enfuvirtide for blocking HIV-1 infection in cultured primary target cells that were derived from either HIV-susceptible hCD4/hCCR5-transgenic rats or healthy human donors.

Activated primary T cells were pretreated with different concentrations of efavirenz, challenged with an HIV-1_{NL4-3} EGFP reporter virus, and analyzed for early viral gene expression on day 3 postinfection. In T cell cultures from both species, the relative percentage of HIV-1-infected cells decreased in a concentration-dependent manner (Fig. 1 *A* and *B*), and the effective concentration, 50% (EC_{50}), and EC_{80} values for efavirenz were in the nanomolar range and statistically indistinguishable for rats and humans (Table 1). Similarly, the EC_{50} and EC_{80} values for efavirenz on HIV-1-infected spleen-derived macrophages from double-transgenic rats and human monocyte-derived macrophages were quite comparable (Fig. 1 *C* and *D* and Table 1). The infection of rat macrophages by a vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-2_{ROD-A} EGFP reporter virus or an HIV-1_{NL4-3}-based reporter virus carrying multiple NNRTI resistance mutations in the reverse transcriptase gene could not or only very inefficiently be inhibited by efavirenz, respectively (Table 1), consistent with analogous studies in human cells (16).

To test the antiviral *ex vivo* efficacy of the entry inhibitor enfuvirtide, a synthetic peptide corresponding to a region in the transmembrane subunit of the HIV-1 envelope glycoprotein (17), we performed a virion-fusion assay (18, 19). Cells were exposed to enfuvirtide and then challenged with HIV-1_{YU-2} virions carrying β-lactamase-Vpr chimeric fusion proteins (BlaM-Vpr). The change in fluorescence emission of the cell-permeable CCF2 substrate after cleavage by BlaM-Vpr, which enters the target cell cytoplasm by virion fusion, is a sensitive marker for virus entry and can be quantified by flow cytometry. The dose–response curves for enfuvirtide-mediated inhibition of HIV-1 entry in T cells from hCD4/hCCR5-transgenic rats and human donors almost superimposed (Fig. 2*B*), and as a result, the EC_{50} and EC_{80} values were very similar (Table 1). This virion-fusion assay could also be adapted to primary macrophage cultures (Fig. 2*A*), and the results obtained demonstrated a similar antiviral potency of enfuvirtide in these HIV-susceptible phagocytic scavengers from both species (Fig. 2*C* and Table 1). In summary, the inhibitory efficacies of a prototypic NNRTI and of a fusion inhibitor in primary HIV target cells from hCD4/hCCR5-transgenic rats and humans are comparable.

Efavirenz Inhibits HIV-1 Infection in hCD4/hCCR5-Transgenic Rats *in Vivo*. Next, we investigated the antiviral *in vivo* potency of the NNRTI efavirenz in HIV-susceptible transgenic rats. Efavirenz was

Table 1. Comparable anti-HIV efficacy of the NNRTI efavirenz and of the fusion inhibitor enfuvirtide in cultured primary cells from hCD4/hCCR5-transgenic rats and humans

		Inhibitory concentration, nM			
		Macrophages		T cells	
Drug and species	Virus	EC_{50}*	EC_{80}*	EC_{50}*	EC_{80}*
Efavirenz					
Human	HIV-1_{NL4-3}	6 (1)	20 (1)	24 ± 14 (3)	93 ± 57 (3)
Rat	HIV-1_{NL4-3}	13 ± 6 (4)	50 ± 17 (4)	21 ± 13 (2)	112 ± 83 (2)
Rat	HIV-1_{NL4-3} RT_{mut}	3,100 ± 600 (2)	15,000 ± 5,000 (2)		
Rat	HIV-2_{ROD-A}	>50,000 (2)	>50,000 (2)		
Enfuvirtide					
Human	HIV-1_{YU-2}	76 ± 65 (2)	352 ± 308 (2)	144 ± 32 (2)	434 ± 99 (2)
Rat	HIV-1_{YU-2}	32 ± 7 (2)	227 ± 135 (2)	116 ± 3 (2)	460 ± 105 (2)

*EC_{50} and EC_{80} values were derived from the dose–response experiments described in Figs. 1 and 2 and from experiments conducted in an analogous manner. Shown is the arithmetic mean ± SEM from independent experiments performed employing cells from one to four donors (shown in parentheses); each infection was done in triplicate.

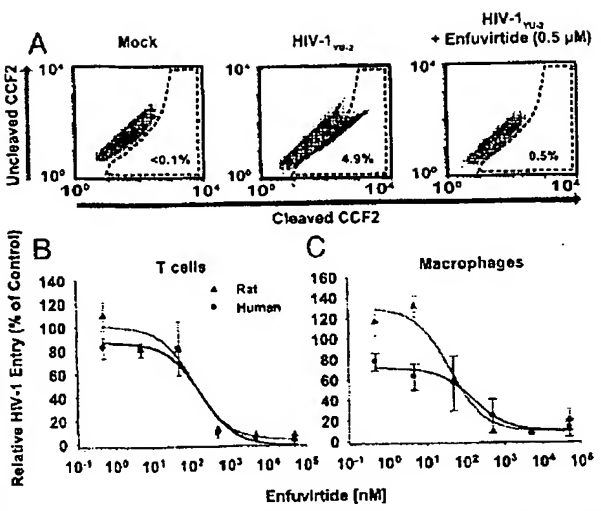

Fig. 2. HIV-1 virion fusion is efficiently blocked in T cells and macrophages from hCD4/hCCR5-transgenic rats by enfuvirtide. (*A*) HIV-1_{YU-2} virion fusion in rat macrophages was analyzed by multiparameter flow cytometry as described in *Materials and Methods*. Shown are representative FACS dot plots for the detection of CCF2 substrate cleavage in rat macrophages which were either mock-infected (*Left*) or HIV-1_{YU-2}-infected either in the absence (*Middle*) or presence (*Right*) of enfuvirtide. (*B* and *C*) Dose–response curves on T cells (*B*) and macrophages (*C*) from both species. The percentage of infected cells in untreated control cells was set at 100%. Two experiments were performed, each in triplicate. EC_{50} and EC_{80} values were determined by using Prism software and are shown in Table 1.

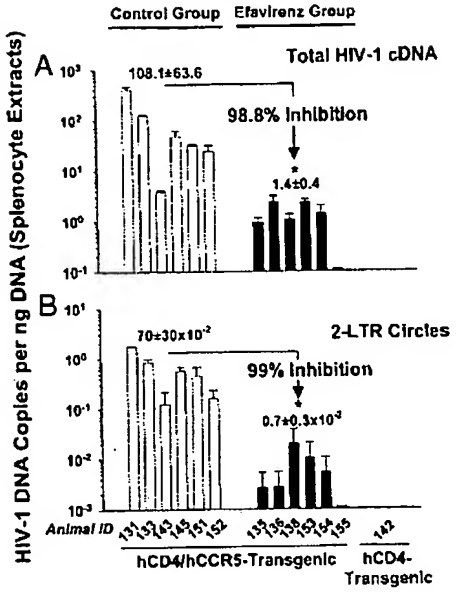

Fig. 3. Efavirenz inhibits HIV-1 infection *in vivo*. hCD4/hCCR5-transgenic rats were treated with either efavirenz at 25 mg/kg per day (efavirenz group) or PBS (control group) by once-daily oral gavage for 3 days. On day 3, animals from both experimental groups as well as a hCD4-single transgenic rat (rat 142) were challenged i.v. with HIV-1_{YU-2}. Dosing was continued in both treatment groups for 4 more days, and then all animals were killed, and their spleens were removed. The antiviral efficacy was assessed by determining the load of either total HIV-1 proviral DNA (*A*) or HIV-1 2-LTR circles (*B*), relative to a rat GAPDH standard in DNA extracts from splenocytes by duplex PCR. Results given for animal groups are the arithmetic mean ± SEM.. Nonparametric statistical analyses were performed by using the Mann–Whitney *U* test; the asterisks indicate $P < 0.0025$.

administered to six hCD4/hCCR5-transgenic rats at a weight-adjusted, pediatric dose of 25 mg/kg per day by once-daily oral gavage. Six control group animals received an equivalent volume of PBS. Rats were dosed 3 days before and 4 days after tail vein challenge with HIV-1_{YU-2}. On day 7, animals were killed, and the HIV-1 cDNA load in DNA extracts from splenocytes was determined by quantitative duplex PCR analysis.

HIV-1 cDNA was readily detected in splenocyte extracts from PBS-treated, HIV-1_{YU-2}-challenged hCD4/hCCR5-transgenic rats, ranging from 3 to 414 HIV-1 cDNA copies per ng of DNA (Fig. 3*A*). As a specificity control, no HIV-1 cDNA could be amplified from samples of a hCD4-single transgenic animal (animal 142) challenged with the identical infectious dose, demonstrating that the amplified HIV cDNA in samples from double-transgenic rats had been generated *de novo* after a receptor complex-mediated infection *in vivo*. Importantly, oral efavirenz treatment had a drastic antiviral activity: the HIV-1 cDNA load in splenocytes from HIV_{YU-2}-infected animals from the efavirenz group was reduced by 98.8% (1.92 $\log_{10}$) relative to the control group (Fig. 3*A*). This antiviral activity was highly significant according to the Mann–Whitney *U* test ($P < 0.0025$). To confirm this result independently, DNA extracts from splenocytes were reanalyzed in a second duplex PCR, this time amplifying circularized HIV-1 cDNA genomes containing two joined LTRs. The presence of these 2-LTR circles in a cell is an established surrogate for successful HIV entry, reverse transcription, and nuclear import (20). In good agreement with the results for total proviral DNA, splenocyte samples from the efavirenz group showed a concordant reduction in 2-LTR circles by 99.0% (2.0 $\log_{10}$, $P < 0.0025$) (Fig. 3*B*). Thus, HIV-1-susceptible hCD4/hCCR5-transgenic rats allowed a rapid and quantitative drug testing, and they demonstrated a high antiviral activity of an orally bioavailable reverse transcriptase inhibitor at a pediatric dosage.

Prophylactic Treatment with Enfuvirtide Markedly Diminishes the Proviral Load, but Its *in Vivo* Potency Depends on the Dosing Regimen. The antiviral efficacy of the entry inhibitor enfuvirtide in hCD4/hCCR5-transgenic rats was tested in an experimental setup analogous to the efavirenz study. In two treatment groups, enfuvirtide was administered by s.c. injection at 4 mg/kg per day, corresponding to the dosage generally applied in HIV-infected children (21). The first group of rats received this daily enfuvirtide dosage divided into two injections at ≈12-h intervals, as recommended in humans, whereas the second group was injected only once daily. All rats were challenged i.v. on day 3 with HIV-1_{YU-2} at a 10-fold lower infectious dose than was used in the efavirenz study.

The twice-daily enfuvirtide treatment group showed a >10-fold reduction (92.8%; 1.14 $\log_{10}$) of the HIV-1 cDNA load in splenocyte extracts relative to the water-injected control group (Mann–Whitney *U* test; $P < 0.018$) (Fig. 4), demonstrating a high antiviral potency of a prophylactic application of the fusion inhibitor in this small animal model. In contrast, the reduction of the HIV-1 cDNA load in the once-daily treatment group was only modest (≈2-fold) and did not reach statistical significance ($P = 0.33$). Again, no HIV-1 cDNA could be amplified from samples of the hCD4-single transgenic animal (animal 53) (data not shown). In summary, enfuvirtide treatment markedly diminished the proviral load in a secondary lymphoid organ in HIV-1-infected hCD4/hCCR5-transgenic rats, but the antiviral *in vivo* potency of the synthetic peptide strongly depended on the dosing regimen.

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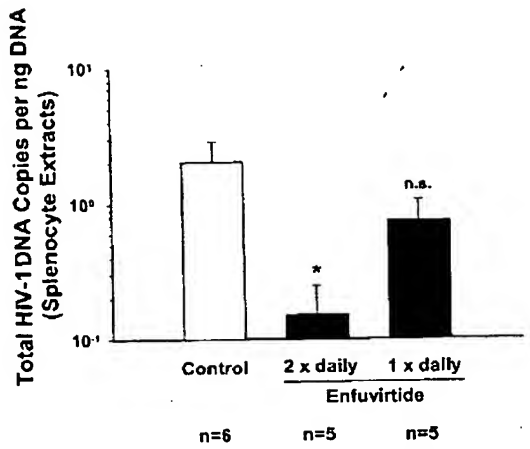

Fig. 4. Enfuvirtide blocks HIV-1 entry *in vivo*. hCD4/hCCR5-transgenic rats were treated s.c. with either enfuvirtide at 4 mg/kg per day either twice daily or once daily. The control group was treated twice daily with distilled water. On day 3, animals from all three groups and one hCD4-single transgenic rat (rat 53; data not shown) were challenged i.v. with HIV-1_{YU-2}. Dosing was continued, and the experiment was terminated on day 4 postinfection. The antiviral efficacy of enfuvirtide was assessed by duplex PCR as described for Fig. 3*A*. Results given for animal groups are the arithmetic mean ± SEM. Nonparametric statistical analyses were performed by the Mann–Whitney *U* test; the asterisk indicates $P < 0.018$; n.s., not significant ($P = 0.33$).

Discussion

Information on the antiviral *in vitro* and *in vivo* potency, toxicity, and pharmacological properties, including bioavailability and pharmacokinetics, guides the development and advancement of anti-HIV compounds. A rapid preclinical validation in a readily available HIV-susceptible small animal would facilitate an evidence-based prioritization of compounds for phase I clinical trials. In this work, we provide proof of principle that rats, which transgenically express the HIV-1 receptor complex on biologically relevant target cells, recapitulate the antiviral potency and basic pharmacological properties of two different anti-HIV drugs that are widely used in HIV-infected patients. Both the orally administered NNRTI efavirenz and the s.c.-injected peptidic fusion inhibitor enfuvirtide had a high antiviral efficacy in this small animal model.

First, the potencies of these prototypic anti-HIV drugs on cultured primary T cells and macrophages from transgenic rats and humans were comparable, with EC_{50} and EC_{80} concentrations differing by no more than 3-fold. Similarly, rat cells revealed a lack of antiviral efficacy of the NNRTI when a drug-resistant HIV-1 or HIV-2 strain was tested. On the level of HIV-infected primary cells *ex vivo*, this observation indicates conserved modes of drug metabolism and action and supports the validity of a rat–human cross-species extrapolation of drug efficacy also in an *in vivo* context. Second, the overall reduction in the spleen-associated HIV cDNA load achieved by enfuvirtide or efavirenz treatment in a 7-day trial in the transgenic rat model, 1.14 $\log_{10}$ and 1.92 $\log_{10}$, respectively, was within the range seen for the reduction in plasma viral load in monotherapy studies in HIV-infected adults. Kilby and colleagues (17) reported that patients receiving 200 mg of enfuvirtide per day by twice-daily injection experienced a ≈1.6 $\log_{10}$ median decline in plasma HIV RNA in a 7-day dosing period (17). Similarly, oral efavirenz monotherapy in an HIV-infected patient at 200 mg per day for 7 days resulted in a ≈1.5 $\log_{10}$ decline in HIV RNA in plasma (22). Although the cross-species comparison for the antiviral *in vivo* potency of these two drugs is promising, the testing of additional compounds in the transgenic rat model will be required to define more carefully the accuracy of its predictive value for HIV-infected humans.

Outbred Sprague–Dawley rats are the most widely used and best validated small animal model for basic studies on bioavailability, pharmacokinetics, and pharmacodynamics of virtually all drug candidates, and pharmaceutical companies use these rodents for large proportions of their mandatory toxicity testing (23). This fact greatly enhances the utility of the HIV-susceptible transgenic rats for preclinical drug efficacy studies. Furthermore, the antiviral *in vivo* efficacy of the synthetic enfuvirtide peptide in hCD4/hCCR5-transgenic Sprague–Dawley rats was strongly dependent on the dosing regimen; a once-daily administration was markedly inferior (≈5-fold lower inhibition) in its antiviral potency compared with a twice-daily regimen. This result is consistent with the known unfavorable pharmacokinetic properties of enfuvirtide, with a short median half-life of the peptide in rats as well as in humans (median $t_{1/2}$ = ≈2 h) (17). The results from the two treatment groups in our enfuvirtide study suggest that unfavorable pharmacokinetic properties affecting the trough concentration of a compound can be reflected in a loss of antiviral efficacy, which further validates the model. In principle, established pharmacokinetic analyses could be conveniently conducted in the course of an efficacy evaluation because repeated blood draws of up to 1.5 ml are possible in HIV-infected rats. Thus, a comprehensive preclinical evaluation of anti-HIV compounds, addressing antiviral *in vivo* potency with a dynamic range of up to 3 orders of magnitude as well as toxicity and pharmacokinetics, can be performed in hCD4/hCCR5-transgenic rats in <2 weeks. As an additional advantage over current xenotransplant models, HIV-susceptible hCD4/hCCR5-transgenic rats harbor both CD4 T cells and macrophages in a physiological frequency, organ distribution, and activation state. Notably, in HIV-infected patients these major HIV target cell populations provide distinct milieus with respect to the effectiveness of antiretroviral therapy (24–27). Furthermore, because of the ease of breeding and handling large numbers of these immunocompetent transgenic rats, even medium-throughput *in vivo* drug screening approaches appear feasible. Despite only transient HIV-1 replication *in vivo* (13), the current study suggests that the hCD4/hCCR5-transgenic rat model is well suited to evaluate new antiviral lead compounds targeting viral entry or reverse transcription, and they may thus help guide the selection of effective antiviral compounds to treat HIV-1 disease in humans. In addition, the testing of alternative anti-HIV strategies, such as integrase inhibitors as well as gene therapy approaches, can be envisioned. Building on such exciting applications for the current form of the model, we are now pursuing different strategies, including virus modifications, adjuvant pharmacostimulation, and additional genetic manipulations of the host, to enhance HIV replication in this rodent model further.

Materials and Methods

Transgenic Rat Model. The generation and initial characterization of hCD4/hCCR5-transgenic rats has been reported in ref. 13. Animal experiments were conducted according to the German Animal Welfare Act and with authorization of the Regierungspräsidium Karlsruhe (35-9185.81/G-100/02); experiments were supervised by animal welfare officers of Heidelberg University.

Primary Cells. Cultures of primary T lymphocytes and macrophages from transgenic rats and random human donors were generated as reported in refs. 12, 28, and 29.

Virus Stocks. The HIV-1_{YU-2} proviral DNA was cloned directly from brain tissue of a patient who died of AIDS dementia complex (30). The generation of replication-competent HIV-1_{YU-2} stocks for *in vivo* infection studies has been reported in ref. 13. Virus stocks were characterized for p24 concentration and for infectious titer (TZM-BL IU) as described in ref. 29. The molecular clones HIV-1_{NL4-3} E^- EGFP, which carries an *egfp*

gene within the *nef* locus (31), HIV-2_{ROD-A} E^- EGFP (32), or HIV-1_{NL4-3} RT_{mut}(K20R, K32R, V35L, K65R, L100I, K103N, L214F, P272A, I293V) E^- EGFP (kind gift from Hans-Georg Kräusslich, University of Heidelberg, Germany) were pseudotyped with VSV-G as reported in ref. 12. HIV-1_{YU-2} virions containing BlaM-Vpr were produced by triple-transfection of 293T cells by calcium phosphate DNA precipitation (18). Two days posttransfection, the supernatant was concentrated by using Centricon Plus-70 spin columns (Millipore, Billerica, MA), and then virus particles were purified through a 20% sucrose cushion (27,000 × *g*, 4°C, 60 min). The virion-enriched pellet was resuspended in medium and stored at −80°C.

Antiviral Drugs. Enfuvirtide (Roche, Indianapolis, IN) was freshly dissolved in distilled H_2O at 9 mg/ml and further diluted in H_2O. Efavirenz (Bristol–Myers Squibb, Jacksonville, FL) was purchased as a drinking solution at 30 mg/ml and diluted in PBS.

***Ex Vivo* HIV-1 Virion-Fusion Assay.** The flow cytometry-based HIV-1 virion-fusion assay was conducted as described in refs. 18 and 19. Briefly, primary T cells and macrophages were pretreated with the indicated concentrations of enfuvirtide for 30 min. Subsequently, cells were challenged with HIV-1_{YU-2} BlaM-Vpr virions (50 ng per 2×10^6 T cells or per $\approx 2 \times 10^5$ macrophages) for 3–4 h, washed, and then loaded with CCF2/AM dye overnight. Fusion was monitored with a three-laser BD FACSAria Cell Sorting System (BD Biosciences, San Jose, CA).

***Ex Vivo* Efficacy Testing of Efavirenz.** Macrophages were pretreated overnight and T cells for 1 h with efavirenz at the indicated concentrations, and then they were subsequently challenged with single-round VSV-G HIV-1_{NL4-3} EGFP reporter virions (200–500 ng of p24 per 3×10^6 T cells or per $\approx 2 \times 10^5$ macrophages). On day 3 postinfection, the percentage of EGFP-positive T cells and macrophages was scored by flow cytometry on a FACSCalibur by using BD CellQuest Pro 4.0.2 software (BD PharMingen, San Jose, CA).

***In Vivo* Efficacy Testing.** Two independent efficacy studies with a similar design were performed (animal age, 8 weeks; weight range, 140–306 g). For the NNRTI study, groups consisting of six hCD4/hCCR5-transgenic rats were treated with either efavirenz at 25 mg/kg per day or PBS by once-daily oral gavage for 3 days. On day 3, rats from both experimental groups were anesthesized and challenged by tail vein injection through a plastic catheter with HIV-1_{YU-2} (6.7×10^6 TZM-BL IU; 5,000 ng of p24 per rat) as described in ref. 13. For the fusion inhibitor study, groups consisting of five or six hCD4/hCCR5-transgenic rats were treated with enfuvirtide at 4 mg/kg per day, applied by either twice-daily or once-daily s.c. injection. The control group was injected twice daily with an equivalent volume of distilled water. On day 3, all rats were challenged i.v. with HIV-1_{YU-2} (6.7×10^5 TZM-BL IU; 500 ng of p24 per rat). In both efficacy studies, one HIV-nonsusceptible, hCD4-single transgenic rat was challenged with the identical virus inoculum. Dosing was continued for 4 more days postchallenge, and then all animals were killed. Total DNA was prepared from single-cell suspensions of splenocytes by using DNeasy tissue kits (Qiagen, Valencia, CA), and it was analyzed by quantitative duplex PCR.

Quantification of HIV-1 DNA Species. The amount of total HIV-1 cDNA and HIV-1 2-LTR circles in splenocyte extracts was analyzed by quantitative duplex PCR by using the ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA). Total HIV-1 cDNA was amplified by using a primer pair specific for *LTR U5* and *gag* and the probe 5′-[fluorescein (FAM)]-CAGTGGCGCCCGAACAGGGA-[rhodamine (TAMRA)]-3′ as reported in refs. 33 and 34. For quantification of 2-LTR circle junctions a forward primer annealing at *U5*, a reverse primer annealing at *U3* and the probe 5′-(FAM)-TCCACACTGACTAAAAGGGTCTGGGGATCTCT-(TAMRA)-3′ were used (33, 34). For standard curves, dilutions of pHIV-1_{NL4-3} E^- EGFP and pU3U5 covering 5 logs were used, supplemented with DNA from uninfected cells. Results obtained for HIV-1 cDNA species were normalized to the amount of cellular DNA, which was quantified in the same reaction by amplification of the rat GAPDH gene DNA (reagents from Applied Biosystems). For the latter, dilutions of genomic DNA extracted from primary rat T cells were used for standard curves. The cycling program was as follows: 2 min at 50°C; 10 min at 95°C; 40 cycles of 15 s at 95°C then 1 min at 60°C. All samples were run in duplicate. Data analysis was performed by using the 7500 system software. The lowest detection limit ranged from 0.02 to 0.03 copies per ng of DNA and from 0.05 copies per ng of DNA for total HIV-1 cDNA and for 2-LTR circles, respectively. Some values for extracts of the treatment groups had to be extrapolated.

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